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(54) **Amplification processes.**

(57) A method for the amplification of a target nucleotide sequence using a first primer comprising 1) a target binding nucleotide moiety which is substantially complementary to the desired portion of the target nucleotide sequence; and 2) a polynucleotide tail; the first primer being such that whilst an extension product thereof may itself serve as a template for primer extension to form an amplification primer extension product, formation of an amplification primer extension product comprising a sequence complementary to the polynucleotide tail is inhibited.

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## AMPLIFICATION PROCESSES

The present invention relates to amplification processes, primers for use in such processes and kits comprising such primers. In particular the invention relates to processes for preparing amplification products having a polynucleotide tail, which tail may for example be employed for solid phase capture or for linkage to a labelling or signalling system. The present invention also relates to primers for use in the amplification processes of the invention and to kits for performing such processes.

Processes for amplifying a desired specific nucleic acid sequence are known and have been described in the literature. K. Kleppe et al in J. Mol. Biol., (1971), 56, 341-361 disclose a method for the amplification of a desired DNA sequence. The method involves denaturation of a DNA duplex to form single strands. The denaturation step is carried out in the presence of a sufficiently large excess of two nucleic acid primers which hybridise to regions adjacent to the desired DNA sequence. Upon cooling two structures are obtained each containing the full length of the template strand appropriately complexed with primer. DNA polymerase and a sufficient amount of each required nucleoside triphosphate are added whereby two molecules of the original duplex are obtained. The above cycle of denaturation, primer addition and extension are repeated until the appropriate number of copies of the desired DNA sequence is obtained. It is indicated that adjustment of the primer concentration may be required.

The above method is now referred to as polymerase chain reaction (PCR) as claimed in United States patents nos. 4683195 and 4683202 wherein amplification of a given nucleic acid sequence on a template is effected by extension of a nucleic acid primer in the presence of *Thermus aquaticus* (Taq) DNA polymerase or the Klenow fragment of *E.coli* DNA polymerase I. The amplification procedure is generally repeated for up to about 50 cycles. The examples provided only relate to short DNA sequences, generally of a few hundred base pairs.

Amplification processes such as the PCR reaction provide a useful tool for amplifying nucleic acid sequences, but it is commonly desirable that the amplified product be capable of attachment to a further species for example for capture or signalling purposes. The further species may therefore be for example a solid phase or signalling moiety. Thus for example it may be desirable to isolate the amplified product, for example as a purification step in the production of nucleotide probes or as a quality control step in their production or in assay methods such as for example in the diagnosis of genetic disorders. It may also be desirable to label or mark, or augment the label or marker associated with or carried by the amplified product.

In order to render the amplified product capable of attachment to a further species the primer(s) used for amplification may for example carry an antibody or antigen, for example, for capture onto a solid phase carrying corresponding antigen or antibody, but such an antibody and antigen would need to be thermostable to withstand PCR temperature cycling. An alternative technique would be to use a primer carrying one member of a complex forming pair such as biotin, the further species carrying the other member of the complex forming pair such as avidin. Such techniques tend to be unsatisfactory because these techniques inter alia, are not readily adaptable to the simultaneous performance of more than one test. An assay in which attachment of the amplified product to the further species was based on nucleic acid hybridisation would overcome at least in part the above-identified problems but if a primer for a PCR reaction were provided with a polynucleotide tail, for example for binding to solid phase immobilised DNA, the Taq DNA polymerase present would cause amplification of the polynucleotide tail as well as the extended target binding portion of the primer. In such a case, where the polynucleotide tail is itself amplified, competition for attachment to the further species (for example to a solid phase or signalling moiety) arises which thereby inhibits binding. The present invention is based on the discovery that the above-identified problems may be overcome, at least in part, if the amplification process is effected using a first primer comprising 1) a target binding-nucleotide moiety which is substantially complementary to the desired portion of the target nucleotide sequence; and 2) a polynucleotide tail; the first primer being such that whilst an extension product thereof may itself serve as a template for primer extension to form an amplification primer extension product, formation of an amplification primer extension product comprising a sequence complementary to the polynucleotide tail is inhibited.

Thus according to one feature of the present invention there is provided a method for the amplification of a target nucleotide sequence, which method comprises contacting the target nucleotide sequence under hybridising conditions, together or sequentially, with a first primer for a desired portion thereof, a corresponding amplification primer, appropriate nucleotides and an agent for polymerisation of the nucleotides the first primer comprising:-

- 1) a target binding nucleotide moiety which is substantially complementary to the desired portion of the

target nucleotide sequence;  
and 2) a polynucleotide tail;

such that the first primer may be subjected to primer extension whereby a first primer extension product is synthesised based on the target polynucleotide sequence as template, and after denaturation of the first  
5 primer extension product from its template and hybridisation of the amplification primer to the desired portion of the first primer extension product, primer extension may be effected to form an amplification primer extension product, the presence of the first primer in the first primer extension product being effective to inhibit formation of a sequence complementary to the polynucleotide tail in the amplification primer extension product.

10 It will be appreciated that the steps of separating primer extension product (whether the extension product be first primer extension product or amplification primer extension product) from its template and contacting the single stranded molecules thus obtained with first primer and amplification primer under conditions such that further primer extension products are synthesised may be repeated as many times as necessary to obtain the desired level of sequence amplification.

15 The first primer may if desired comprise the target binding nucleotide moiety and the polynucleotide tail with a nucleotide polymerisation blocking moiety therebetween. The presence of a nucleotide polymerisation blocking moiety is not however essential since the agent for polymerisation may be inhibited from forming an extension product based on the polynucleotide tail as template for example if more than one polynucleotide tail is bonded to the target binding nucleotide moiety or for example if the target binding  
20 nucleotide moiety and the polynucleotide tail are linked to one another but the moiety and the tail are in the opposite sense to one another, the target binding nucleotide moiety generally being in the 5'→3' sense and the polynucleotide tail being therefore generally in the 3'→5' sense, with linkage via their 5' termini.

If desired the amplification primer may, in addition to the first primer, comprise:-

1) a target binding nucleotide moiety which is substantially complementary to the desired portion of the  
25 target nucleotide sequence; and

2) a polynucleotide tail; such that the amplification primer may be subjected to primer extension whereby an amplification primer extension product is synthesised based on the first primer extension product as template, and after denaturation of the amplification primer extension product from its template and hybridisation of the first primer to the desired portion of the amplification primer extension product,  
30 primer extension may be effected to form a first primer extension product, the presence of the amplification primer in the amplification primer extension product being effective to inhibit formation of a sequence complementary to the polynucleotide tail in the first primer extension product.

As stated in relation to the first primer, a nucleotide polymerisation blocking moiety may if desired be positioned between the target binding nucleotide moiety and the polynucleotide tail although this not  
35 necessary.

The method of the present invention may, for example, be employed to capture amplified product for example on to solid phase and/or to bind the amplified product to label or marker, regardless of whether the amplified product itself carries a label or marker. Thus the method of the present invention enables the signal associated with an amplified product to be enhanced and moreover renders an amplified product  
40 capable of detection and/or capture on to solid phase by any convenient technique such as for example techniques as described in European Patent Publication Nos. 79,139; 124,221; 128,332; 153,873; 185,494; 204,510; or 225,807 as described in U.K. Patent Publication Nos. 2,019,408 or 2,169,403 or as described in PCT Patent Publications W087 03622 or W088 02785. Furthermore the method of the present invention enables more than one amplification product to be identified in a single test by appropriate design of the  
45 polynucleotide tail. Thus for example the polynucleotide tail of a primer in respect of a first locus may be distinguishable from the polynucleotide tail in respect of a further locus or loci such that amplified product in respect of the first locus may for example be captured onto a first and distinguishable solid phase whilst amplified product(s) in respect of a further locus or loci are captured on one or more further solid phase(s) which solid phases are preferably distinguishable from one another. Similarly if desired, amplified product in  
50 respect of a first locus may be distinguishably labelled or marked via the primer's polynucleotide tail whilst amplified product(s) in respect of a further locus or loci may also be labelled or marked preferably such that amplified product in respect of each locus may be distinguished by a different label or marker. Thus for example the polynucleotide tail may be employed to match a given signal to a given locus.

The method of the present invention may also be effected in the solution phase, conveniently using an  
55 oligonucleotide primer which is essentially complementary to the 3' end of the non-amplifiable tail primer as far as the nucleotide polymerisation blocking moiety and which is for example conjugated to a hapten capable of isolating unincorporated non-amplifiable tail primer. The preferred hapten is a magnetic bead. After contacting the reaction mixture with the hapten conjugated oligonucleotide and placing this for

example in a magnetic field, the unincorporated non-amplifiable tail primer is retained, preferably within a microtitre dish well. The reaction mixture including unincorporated non-amplifiable tail primers is then further contacted under hybridising conditions with a detection primer conjugated, for example, to a fluorophore. Any convenient fluorophore may be used, such as fluorescein and rhodamine, for example  
 5 fluorescein. The presence of the amplified product may then be detected for example by fluorescence polarisation according to the methods described in our European Patent Application No. 90301135.1. The hybridised complex is conveniently excited with ultra violet light, preferably at 495nm wavelength when fluorescein is the chosen fluorophore or preferably at 554nm when rhodamine is the chosen fluorophore. Whilst we do not wish to be bound by theoretical considerations it is believed that the solution phase  
 10 embodiments outlined above are not suitable for use with the single stranded products of linear amplification.

The method of the present invention is applicable to all areas of diagnostic medicine and other diagnostic sciences, for example forensic, agricultural, veterinary, food sciences or molecular biology research where it is necessary to detect or measure specific nucleic acid sequences. In particular it is  
 15 applicable to the detection of infectious micro-organisms and to the detection of point mutations, gene deletions and rearrangements which give rise to various inherited diseases, such as for example cystic fibrosis, alpha-1-antitrypsin deficiency and predisposition to disease.

In this regard the method of the present invention may advantageously be employed in conjunction with the allele specific amplification method referred to as the Amplification Refractory Mutation System (ARMS)  
 20 which is described and claimed in our European Patent Application No.89302331.7, Publication No. 0332435 and additionally described by Newton et al in Nucleic Acids Research 17 (7) 2503-2516 (1989). The method as described in Nucleic Acids Research may also be effected by linear amplification or by other amplification techniques as opposed to the polymerase chain reaction as described and claimed in our above-mentioned European Patent Application. ARMS uses primers that allow amplification in an allele  
 25 specific manner. Allele specificity is provided by the complementarity of the 3'-terminal base of a primer with its' respective allele. Amplification is inhibited when the 3' terminal base of the primer is mismatched. Where specificity of a given primer is not absolute, this can be achieved by destabilising its 3'-terminus by including an additional mismatched base. Both ARMS and PCR can be performed on small amounts of crude DNA from biological samples and therefore have considerable diagnostic utility.

Allele specific amplification as outlined above may conveniently be effected using a single solid phase  
 30 for a reaction to detect the presence of both normal and variant alleles of a genetic locus. For a given locus, for example the S locus of the alpha-1-antitrypsin gene (AAT-S locus), the internal control signal primers are labelled with a fluorophore such as fluorescein and both ARMS signal primers are labelled with a different fluorophore such as rhodamine. The different colours green (fluorescein), red (rhodamine) and their  
 35 combination yellow (red + green) allow the detection of all normal and variant heterozygotes and homozygotes. By way of example two solid phases bind respectively an internal control capture oligonucleotide plus a capture oligonucleotide for the variant sequence of AAT-S locus as well as a further internal control capture oligonucleotide and a capture oligonucleotide for the normal sequence of the AAT-S locus. Subsequent hybridisation with the ARMS amplification products having non-amplifiable tails allows  
 40 different coloured fluorescent products to be identified. Thus for example a normal individual would show up on the first solid phase as green (fluorescein only), similarly a homozygous S variant individual would show up as green on the second solid phase. A heterozygote would appear yellow on both solid phases (green + red). Similarly a normal individual would show up yellow on the second solid phase and a homozygous S would show up yellow on the first solid phase. The above mentioned aspects are particularly useful in  
 45 respect of dipstick type assay formats. Also a reduction in the number of solid phases required simplifies the chosen assay.

Allele specific amplification as outlined above may alternatively be carried out in the solution phase. Thus for example each unincorporated non-amplifiable tail primer is removed as hereinbefore described. The reaction mixture is then contacted with a plurality of detection primers under hybridising conditions with  
 50 each detection primer being conjugated to a different fluorophore. Any convenient fluorophore such as fluorescein, rhodamine, Texas red or lucifer yellow may be used. The presence of each amplification reaction product is then detected for example using fluorescence polarisation as hereinbefore described.

According to a still further feature of the present invention there is provided a kit for the amplification of a target nucleotide sequence which comprises:-  
 55 a first primer and a corresponding amplification primer for each target nucleotide sequence to be amplified, each first primer comprising:-

- 1) a target binding nucleotide moiety which is substantially complementary to the desired portion of the target nucleotide sequence; and

2) a polynucleotide tail; the presence of the first primer in the first primer extension product being effective to inhibit formation of a sequence complementary to the polynucleotide tail in the amplification primer extension product.

If desired the first primer may additionally include a nucleotide polymerisation blocking moiety, the said moiety being positioned between the target binding nucleotide moiety and the polynucleotide tail.

If desired the amplification primer may also comprise:-

- 1) a target binding nucleotide moiety which is substantially complementary to the desired portion of the target nucleotide sequence; and
- 2) a polynucleotide tail; the presence of the amplification primer in the amplification primer extension product being effective to inhibit formation of a sequence complementary to the polynucleotide tail in a first primer extension product.

If desired the amplification primer may additionally include a nucleotide polymerisation blocking moiety, the said moiety being positioned between the target binding nucleotide moiety and the polynucleotide tail.

The kit of the present invention may, if desired include internal control primers, where appropriate.

The kit of the present invention may, if desired, include appropriate different nucleotides and/or an agent for polymerisation of the nucleotides.

Furthermore, the kit of the present invention may, if desired include solid phase, for binding directly or indirectly to the polynucleotide tails of the primers for example in respect of each target nucleotide sequence to be amplified. Thus for example, the solid phase may carry a nucleotide sequence substantially complementary to the polynucleotide tail of the primer. If more than one target nucleotide sequence is to be amplified in the same reaction vessel, then the solid phase for binding directly or indirectly to the polynucleotide tail of the primer is preferably distinguishable in respect of each target nucleotide sequence to be amplified.

The expression "target nucleotide sequence" as used herein means a nucleotide sequence comprising the sequence to be amplified. Thus for example if the present invention is applied to the diagnosis of  $\beta$ -thalassaemias a sample may contain as many as 60, for example 50, separate potential variant sequences and therefore the sample would contain as many as 60 target nucleotide sequences all of which may, if desired, be amplified (for example as discussed in European Patent Publication No. 237,362) and the amplified products distinguished according to the present invention.

The term "nucleotide" as used herein can refer to nucleotides present in either DNA or RNA and thus includes nucleotides which incorporate adenine, cytosine, guanine, thymine and uracil as base, the sugar moiety being deoxyribose or ribose. It will be appreciated however that other modified bases capable of base pairing with one of the conventional bases, adenine, cytosine, guanine, thymine and uracil, may be used in the diagnostic first primer and amplification primer employed in the present invention. Such modified bases include for example 8-azaguanine and hypoxanthine. If desired the nucleotides may carry a label or marker so that on incorporation into a primer extension product, they augment the signal associated with the primer extension product, for example for capture on to solid phase.

It will be appreciated that where the process of the present invention is to be used to amplify a target nucleotide sequence which does not contain all four different nucleotides, then an extension product of the first primer and if desired, an extension product of the amplification primer may be formed in the presence of only the appropriate corresponding nucleoside triphosphates and all four different nucleoside triphosphates would not be necessary and the expression "appropriate nucleotides" as used herein is to be understood accordingly.

The agent for polymerisation of the nucleotides may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E.coli* DNA Polymerase I, Klenow fragment of *E.coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase, and other enzymes, including thermostable enzymes. The term "thermostable enzyme" as used herein refers to any enzyme which is stable to heat and is heat resistant and catalyses (facilitates) combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and will proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths. There may be enzymes for example, thermostable enzymes, however, which initiate synthesis at the 5' end and proceed in the other direction, using the same process as described above. A preferred thermostable enzyme which may be employed in the process of the present invention is that which can be extracted and purified from *Thermus aquaticus*. Such an enzyme has a molecular weight of about 86,000 - 90,000 daltons as described in European Patent Publication No. 237,362 (see also European Patent Publication No 258,017). *Thermus aquaticus* strain YT1 is available without restriction from the American Type Culture

Collection, 12301 Parklawn Drive, Rockville, Maryland, USA as ATCC 25,104.

The term "primer" is used herein to refer to a binding element which comprises an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of appropriate nucleotides and an agent for polymerisation such as a DNA polymerase in an appropriate buffer ("buffer" includes pH, ionic strength, cofactors, etc.) and at a suitable temperature. In respect of the first primer the oligonucleotide portion of the binding element is termed the "target binding nucleotide moiety".

The term "oligonucleotide" as used herein is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three. Its exact size will depend on many factors, such as the reaction temperature, salt concentration, the presence of denaturants such as formamide, and the degree of complementarity with the sequence to which the oligonucleotide is intended to hybridise.

The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature and source of primer and use of the method. For example, depending on the complexity of the target sequence, the first and amplification primers typically contain 12-35, for example, 15-35 nucleotides capable of hybridisation to the target nucleotide sequence, although they may contain more or fewer such nucleotides. Primers having only short sequences capable of hybridisation to the target nucleotide sequence generally require lower temperatures to form sufficiently stable hybrid complexes with the template.

The term "complementary to" is used herein in relation to nucleotides to mean a nucleotide which will base pair with another specific nucleotide. Thus adenosine triphosphate is complementary to uridine triphosphate or thymidine triphosphate and guanosine triphosphate is complementary to cytidine triphosphate. It is appreciated that whilst thymidine triphosphate and guanosine triphosphate may base pair under certain circumstances they are not regarded as complementary for the purposes of this specification. It will also be appreciated that whilst cytosine triphosphate and adenosine triphosphate may base pair under certain circumstances they are not regarded as complementary for the purposes of this specification. The same applies to cytosine triphosphate and uracil triphosphate.

The primers herein are selected to be "substantially" complementary to the different strands of each specific sequence to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. Commonly, however, the primers have exact complementarity except with respect to analyses effected according to the method described in Nucleic Acids Research 17 (7) 2503-2516, (1989) or a corresponding method employing linear amplification or an amplification technique other than the polymerase chain reaction.

The first primer and if desired the amplification primer comprise a target binding nucleotide moiety (as hereinbefore defined), a polynucleotide tail and, if desired, a polymerisation blocking moiety. The polynucleotide tail can be any chosen sequence, provided

- 1) that it is long enough to provide a stable hybrid with a substantially complementary sequence under given stringency conditions, and
- 2) that it be sufficiently different from the target binding nucleotide moiety, to avoid significant hybrid formation between
  - a) the target nucleotide sequence and the polynucleotide tail; and
  - b) the polynucleotide tail and the target binding nucleotide moiety.

This latter condition is required to prevent molecular confusion with concomitant appearance of false results.

The polynucleotide tail may code for a particular gene product or products, or may code for no gene product at all. Thus, any structural gene or portion thereof could be used as the polynucleotide tail. A preferred sequence, however, would not code for a given gene since such coding may cross hybridise with complementary gene sequences present in the analyte. It is thus preferred to choose a polynucleotide sequence which is non-coding, and not likely to be complementary to sequences in the analyte such as, for example, sequences comprising poly deoxy G, poly deoxy A, poly deoxy GA, poly deoxy GAT, poly deoxy GTA, or any other low complexity (repeating) sequence or any randomly generated sequence. Any such (randomly generated) sequence may be checked against an appropriate nucleotide sequence database to ensure that binding will not be expected to take place between the randomly generated sequence and the

target sequence. The randomly generated sequence may be further checked to ensure that no spurious binding takes place with the target or other sequences in the analyte by dot blot analysis of samples of the analyte using methods known per se.

The term "polynucleotide" as used herein means a sequence of more than one nucleotide (as hereinbefore defined) and thus includes the term "oligonucleotide" (as hereinbefore defined).

The term "polymerisation blocking moiety" as used herein means any moiety which when linked for example covalently linked, between a first nucleotide sequence(s) and a second nucleotide sequence(s) is effective to inhibit and preferably prevent, more preferably completely prevent amplification (which term includes any detectable copying beyond the polymerisation blocking moiety) of either the first or the second nucleotide sequence(s) but not both such first and second sequences. In general an agent for polymerisation will initiate polynucleotide synthesis at the 3' end of a primer and will proceed in the 5' direction along the template strand and thus in general the polynucleotide tail will be 5' of the polymerisation blocking moiety and the target binding nucleotide moiety will be 3' of the polymerisation blocking moiety. Where however an agent for polymerisation is to be used which initiates synthesis at the 5' end of a primer and proceeds in the 3' direction along the template strand, then the target binding nucleotide moiety will be 5' of the polymerisation blocking moiety and the polynucleotide tail will be 3' of the polymerisation blocking moiety. Preferably the polymerisation blocking moiety comprises a moiety inert under the conditions of the method of the invention.

If desired the target binding nucleotide moiety may carry more than one polynucleotide tail and/or the polynucleotide tail(s) may carry more than one target binding nucleotide moiety. In such circumstances the presence of a polymerisation blocking moiety between the polynucleotide tail(s) and the target binding nucleotide moiety (moieties) may be unnecessary as for example disclosed in European Patent application No. 317077. Similarly the target binding nucleotide moiety and the polynucleotide tail may be linked to one another, the moiety and tail however being disposed in the opposite sense to one another. In such a situation, again, the presence of a polymerisation blocking moiety between the polynucleotide tail and the target binding nucleotide moiety may not be necessary. In such a situation the target binding moiety would generally be disposed in the 5'→3' sense and the polynucleotide tail generally disposed in the 3'→5' sense, with linkage via their 5' termini.

Preferably a polymerisation blocking moiety, where present, comprises a moiety inert under the conditions of the method of the invention employed and linked, for example covalently linked, between the polynucleotide tail and the target binding nucleotide moiety. A wide range of such moieties may be envisaged for this purpose as exemplified below. For example the polymerisation blocking moiety may comprise a bead, for example a polystyrene, glass or polyacrylamide bead or the polymerisation blocking moiety may comprise a transition metal such as for example iron, chromium, cobalt or nickel (for example in the form of a transition metal complex with the polynucleotide tail and the target binding nucleotide moiety) or an element capable of substituting phosphorus such as for example arsenic, antimony or bismuth linked between the polynucleotide tail and the target binding nucleotide moiety. The blocking moiety might similarly but less preferably involve substitution of the usual phosphate linking groups for example where oxygen is replaced leading to inter alia phosphorodithioates, phosphorothioates, methylphosphonates, phosphoramidates such as phosphormorpholidates, or other residues known per se. Alternative blocking moieties include any 3'-deoxynucleotide not recognised by restriction endonucleases and seco nucleotides which have no 2'-3' bond in the sugar ring and are also not recognised by restriction endonucleases.

The minimum molecular length between the said tail and the target binding nucleotide moiety is generally one atom. The said molecular length is conveniently the equivalent of from 1 to 200 nucleotides, advantageously the equivalent of from 1 to 10 nucleotides, preferably the equivalent of 2-4 nucleotides, for example the equivalent of about 2 or about 4 nucleotides, depending on the nature of the moiety or combination of different moieties.

Thus for example the polymerisation blocking moiety may comprise at least one deoxy ribofuranosyl naphthalene or ribofuranosyl naphthalene moiety, advantageously at least 2 (such as 2 to 10), preferably at least 3 (such as 3 to 8), more preferably at least 4 (such as 4 to 6) such moieties. If desired the deoxyribofuranosyl or ribofuranosyl naphthalene moiety may be linked to the adjacent nucleotides via a 3'-furanosyl linkage or preferably via a 2'-furanosyl linkage. In particular deoxyribofuranosyl naphthalene moieties may be used, linkage to the adjacent nucleotides of the polynucleotide tail and the target binding nucleotide moiety being via a 2'-deoxyribofuranosyl group or more preferably a 3'-deoxyribofuranosyl group. If desired the polymerisation blocking moiety may comprise a straight chain alkylene grouping having one or more carbon atoms, for example at least 2 conveniently at least 3, advantageously at least 4, preferably at least 5, more preferably at least 6 carbon atoms. The upper limit on the number of carbon atoms in the alkylene chain is only determined by synthetic convenience. Thus for example the grouping

may contain from 6 to 20 carbon atoms, which alkylene grouping may if desired carry at least one, for example 1-6 C<sub>1-3</sub> alkyl substituents. The alkylene grouping, or the backbone thereof where substitution is present, conveniently has at least 7, 8, 9, 10, 11 or 12 carbon atoms (the order of preference being 7<8<9<10<11<12) as the minimum number and 19, 18, 17 and 16 carbon atoms (the order of preference being 19<18<17<16) as the maximum number of carbon atoms.

The term "amplification primer" is used herein to refer to a primer which is capable of hybridising to the nucleic acid strand which is complementary to the nucleic acid strand to which the first primer is capable of hybridising, the "amplification primer" having a nucleotide sequence such that it is capable of hybridising to a first primer extension product, after separation from its complement, whereby the first primer extension product serves as a template for synthesis of an extension product of the amplification primer, thereby facilitating amplification.

#### Brief description of the drawings

- Figure 1 is an autoradiograph showing the results of using deoxyribofuranosyl, alkylene and methyl phosphonate polymerisation blocking moieties in primers employed in the method of the present invention.
- Figure 2 is an autoradiograph showing the results of using single deoxyribofuranosyl and methyl phosphonate polymerisation blocking moieties in primers employed in the method of the present invention.
- Figure 3 is an autoradiograph showing the results of using phosphate and phosphoramidate polymerisation blocking moieties in primers employed in the method of the present invention.
- Figure 4 is a photograph showing the results obtained using a microtitre plate test capture system.
- Figure 5 illustrates a convenient assay format for performing the method of the present invention. The illustration shows one well of a microtitre plate with a protein coated internal surface. Immobilised on the internal surface is a solid phase capture oligonucleotide. The free end of this oligonucleotide is hybridised to a unique tail sequence on a double stranded amplification product. The other end of the PCR product likewise comprises a tail sequence which is hybridised to a signal oligonucleotide including an enzyme label represented by E. This label is used to react with a substrate leading to a detectable colour change as represented by the arrow and accompanying asterisk. NN represents a polymerisation blocking moiety which prevents formation of a double stranded tail.
- Figure 6 is a photograph which illustrates the generation of PCR products in an allele specific amplification (ARMS) assay of the S locus of the alpha-1-antitrypsin gene.
- Figure 7 is a photograph which illustrates the microtitre well arrangement for the above ARMS assay of the S locus of the alpha-1-antitrypsin gene and using an internal control based on exon V of the alpha-1-antitrypsin gene.
- The invention is illustrated, but not limited, by the following Examples in which the oligonucleotides (5' - 3') are as stated below:-

#### Oligonucleotide design

The nucleotide primary structures of the non-amplifiable tails were generated using a computer program to generate random nucleotide sequences. The nucleotide sequences of candidate non-amplifiable tails were inspected for guanosine content; particularly G-rich sequences or sequences with more than three consecutive G residues were discarded. The remaining candidates were tested against the primate DNA nucleotide sequences in Genbank version 63.0 (Nucleic Acids Research, 12, 387-395, 1984) allowing 2 mismatches. Candidates that matched were also discarded. All oligonucleotides were synthesised using an Applied Biosystems 380A or 380B DNA synthesiser according to the manufacturer's protocols:-

Oligonucleotide 1

AATTCCGTGCATAAGGCTGTGCTGACCATCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG

Oligonucleotide 2

AATTCCGTGCATAAGGCTGTGCTGNNNNTCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG (wherein N represents a 2'-deoxyribofuranosyl naphthalene moiety, the 5' and 3' terminal naphthalene moieties being linked via the 3'-PO<sub>4</sub> grouping to the adjacent nucleotide).

Oligonucleotide 3

AATTCCGTGCATAAGGCTGTGCTGNNTCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG (wherein N

represents a 3'-deoxyribofuranosyl naphthalene moiety, the naphthalene moieties being linked via the 2'-PO<sub>4</sub> grouping to the adjacent nucleotide thereby introducing a kink into the DNA chain).

Oligonucleotide 4

AATTCGTCGATAAGGCTGTGCTGNNNNTCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG (wherein  
5 N represents a 3'-deoxyribofuranosyl naphthalene moiety, the naphthalene moieties being linked via the 2'-PO<sub>4</sub> grouping to the adjacent nucleotide thereby introducing a kink into the DNA chain).

Oligonucleotide 5

CATGGCCCCAGCAGCTTCAGTCCCTTTCTC

Oligonucleotide 5 is a 30-mer which is complementary to the 3' end of all the oligonucleotides 1-4  
10 described above and oligonucleotides 6 and 8 below; the purpose of the experiment being to see whether oligonucleotide 5 can be extended using the above-described oligonucleotides 1-4 and the below described oligonucleotides 6 and 8 as template.

Oligonucleotide 6

AATTCGTCGATAAGGCTGTGCTGNNNNCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG (wherein  
15 NNNN represents a straight chain alkylene moiety of 16 carbon atoms).

Oligonucleotide 7

TTTTTTTTTTTATCAACTTACTTGCCTATA

this oligonucleotide may be used for attachment to a solid phase via an amino link on the 5' end of the final  
20 T of the poly T region. The sequence of nucleotides 3' of the poly T region is complementary to the polynucleotide tail of an ARMS primer specific for the normal S locus of the alpha-1-antitrypsin gene.

Oligonucleotide 8

AATTCGTCGATAAGGCTGTGCTGNNNNCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG in which N  
represents a 4-methyl phosphonate substituted thymidine residue.

Similarly oligonucleotides 6 and 8 are used as template to see whether oligonucleotide 5 can be  
25 extended.

### Example 1

30 The above-mentioned oligonucleotides were used to demonstrate the failure of Taq DNA polymerase to extend an oligonucleotide primer across a nucleotide polymerisation blocking moiety present in a template DNA strand.

Oligonucleotide 5 (30 p moles) was labelled at the 5' terminal hydroxylgroup using  $\gamma$  <sup>32</sup>P-ATP 75 $\mu$ ci (15 pmoles) (Amersham) and T4 polynucleotide kinase (4 units) in a 50 $\mu$ l reaction volume containing 50nM  
35 Tris.HCl pH7.6, 10mM MgCl<sub>2</sub>, 5mM DTT 100 $\mu$ M spermidine and 100 $\mu$ M EDTA. The kinase reaction was carried out at 37°C for 20 minutes. To completely destroy any remaining kinase activity the reaction mixture was boiled for 5 minutes, followed by a phenol: chloroform (1:1) extraction (37.5  $\mu$ l of each component). This was followed by mixing and centrifugation of the mixture for 2 minutes at 13,500 r.p.m. The aqueous layer was removed and kept for the reactions.

40 Oligonucleotides 1-4, 6, and 8 were purified by acrylamide gel electrophoresis, u.v. shadowing, butan-1-ol concentration, phenol/chloroform extraction and ethanol precipitation. Oligonucleotide 5 was purified by HPLC using an anion exchange column (Mono Q, Pharmacia) equilibrated with Buffer A (10mM NaOH, 0.5M NaCl). The oligonucleotide was eluted with a gradient of Buffer B (10mM NaOH, 0.9M NaCl) 0-100% in 60 minutes. Fractions corresponding to the product peak were pooled and desalted using a PD10 column  
45 (Pharmacia) then concentrated by drying under vacuum. The purity of the oligonucleotides was checked by <sup>32</sup>P labelling using <sup>32</sup>P ATP (Amersham) and T4 polynucleotide kinase followed by polyacrylamide gel electrophoresis under denaturing (7M urea) conditions.

The following reactions were effected:

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				Length of product expected if naphthalene bridge stops enzyme
5	a)	Labelled 5	+ dNTPs (ACGT)	30 No extension as no template
	b)	" 5	+ 1	" -
	c)	" 5	+ 2	"
10	d)	" 5	+ 3	"  No dNTPs
	e)	" 5	+ 4	"
	f)	" 5	+ 6	"
	g)	" 5	+ 8	" -
15	h)	Labelled 5	+ 1 + dNTPs(ACGT)	63 Control
	i)	" 5	+ 2 + dNTPs(ACGT)	35
	j)	" 5	+ 3 + dNTPs(ACGT)	35
20	k)	" 5	+ 4 + dNTPs(ACGT)	35
	l)	" 5	+ 6 + dNTPs(ACGT)	35
	m)	" 5	+ 8 + dNTPs(ACGT)	35

25 If DNA polymerisation is not prevented by the polymerisation blocking moiety the full length oligonucleotide will be copied to give a 63 mer product as expected in control reaction (h). However, if the blocking moiety stops the enzyme (Taq DNA polymerase) then a 35 mer product is expected, this representing chain extension up to the start of the polymerisation blocking moiety.

30 Each reaction contained 1 pmole of labelled oligonucleotide 5 and 3 pmoles of the appropriate long oligonucleotide 1-4, 6 and 8. The dNTPs, where required as shown, were added to a final concentration of 100 $\mu$ M each. There was no denaturation step before 2 units of Taq DNA polymerase (Cetus - Amplitaq) were added to make a final reaction volume of 100 $\mu$ l containing 1.2mM magnesium chloride, 50mM potassium chloride, 10mM Tris HCl (pH 8.3) and 0.01% gelatin (1xbuffer A). The tubes were placed in a 35 91 °C water bath for 2 minutes then at 60 °C for 58 minutes. A 5 $\mu$ l aliquot was taken from each tube. In addition 1 $\mu$ l of labelled oligonucleotide 5 was added to 89 $\mu$ l autoclaved deionised water (Milli Q Standard) and 10 $\mu$ l of 10x buffer A (1x buffer A being as herein defined) and 5 $\mu$ l of this taken to act as a marker for oligonucleotide 5. All the 5 $\mu$ l aliquots were mixed with 5 $\mu$ l formamide dye (80% formamide, 10mM NaOH 1mM EDTA and 0.1% Bromophenol Blue), and denatured by boiling for 5 minutes. The aliquots were 40 electrophoresed on a pre-run (1hr, 500V) 15% denaturing polyacrylamide gel. 8M Urea in 1 x TBE buffer (0.089M TrisBorate, 0.089M boric acid, 0.002M EDTA) for 5 hours at 900V and autoradiographed for two hours at room temperature with X-ray film.

45 The results obtained are shown in the autoradiograph depicted in Figure 1. These demonstrate that the polymerisation blocking moieties present in oligonucleotides 2, 3, 4, 6 and 8 stop Taq DNA polymerase catalysed oligonucleotide extension.

#### Preparation of Starting Materials

##### 50 A) Preparation of 2'-deoxynaphthalene oligonucleotide (2)

##### 1) Preparation of oligodeoxyribonucleotide

55 The fully protected oligodeoxyribonucleotide of sequence (2) was prepared on an Applied Biosystems 380A DNA synthesiser from 5'- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl-N<sup>2</sup>-isobutyryl-2'-deoxyguanosine bound to controlled pore glass via 3'-OH and a succinylglycylglycylaminopropyl spacer (Applied Biosystems Inc) and

the 2-cyanoethyl-N, N-diisopropylaminophosphoramidite of 5'- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl-N<sup>6</sup>-benzoyl-2'-deoxyadenosine, 5'- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl-N<sup>4</sup>-benzoyl-2'-deoxycytidine, 5'- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl-N<sup>2</sup>-isobutyryl-2'-deoxyguanosine, 5'- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl-2'-deoxythymidine (Applied Biosystems Inc) and 1-[2'-deoxy- $\beta$ -D-ribofuranosyl-5'-O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl-3'-(methoxymorpholinophosphine)]naphthalene (prepared as described in (2) below). Alternatively, the fully protected oligodeoxyribonucleotide sequence and those referred to below may be prepared by the manual methods as described by Atkinson and Smith in 'Oligonucleotide Synthesis, a Practical Approach' (M.J. Gait Editor, IRL Press, Oxford, Washington DC, pages 35-81).

1-[2'-deoxy- $\beta$ -D-ribofuranosyl-5'-O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl-3'-(methoxy-N-morpholinophosphine)]naphthalene (70mg) in anhydrous acetonitrile (1ml) was used in place of a normal phosphoramidite at position 5 on a Applied Biosystems 380A DNA synthesiser to introduce the naphthalene substituted residues into the oligonucleotide where indicated by "N" in oligonucleotide 2 above. The procedures consisted briefly of: (1) removal of the  $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl group with 3% trichloroacetic acid in dichloromethane; (2) Coupling of 1-[2'-deoxy- $\beta$ -D-ribofuranosyl-5'-O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl-3'-(methoxy-N-morpholino-phosphine)]naphthalene (0.1M solution) activated by tetrazole for 3 minutes; (3) iodine oxidation of the intermediate phosphite to a phosphate; (4) a capping step with acetic anhydride. The detritylated oligodeoxyribonucleotide sequence was cleaved from the solid support and completely deprotected by treatment with ammonium hydroxide solution (sp.g. 0.88) for 16h. at 55 °C. The ammonium hydroxide solution was evaporated and the residue dissolved in water (1ml). An aliquot of the solution containing the oligodeoxyribonucleotide was purified as follows:-

An aliquot of the crude oligodeoxyribonucleotide (225 $\mu$ l) was precipitated by the addition of 25 $\mu$ l of 3M sodium acetate (pH 5.2), mixing and addition of 750  $\mu$ l of absolute ethanol to the mixture. After further mixing the mixture was left at ambient temperature (25 °C) for one hour and then at 4 °C for a further one hour in order to encourage precipitation of the DNA. The mixtures were centrifuged for 10 minutes at 13,500 rpm and the supernatant removed. The pellets were left to air dry for an hour and then reconstituted with 50  $\mu$ l of formamide dye (80% formamide, 10mM NaOH, 1mM EDTA 0.1% bromophenol blue) at 37 °C for 20 minutes. The sample was boiled for 5 minutes, immediately put on ice and loaded onto a 15% polyacrylamide denaturing gel (7M Urea) in 100mM TrisBorate, 2mM EDTA, pH 8.3 buffer. The gel had been prerun for 1 hour at 500V. The samples were electrophoresed overnight at 50V, that being until the bromophenol dye had run to the bottom of the (0.3 x 25 x 17 cm<sup>3</sup>) gel. The oligodeoxyribonucleotide was visualised by ultraviolet shadowing at 254nm on Merck 5554 20 x 20 aluminium backed thin layer chromatography plates covered with Saran wrap. The product bands were excised using a new scalpel for each oligodeoxyribonucleotide to prevent contamination. The gel slices were placed into 1 inch lengths of treated 1 inch wide dialysis tubing containing 1ml 10mM TrisBorate, 0.2mM EDTA, pH 8.3. Treatment of the tubing consisted of boiling in 1mM EDTA for 10 minutes and rinsing three times by boiling in autoclaved deionised water (Milli Q). The oligodeoxyribonucleotide was eluted from the gel slices by electroelution at 200V for one and a half hours at the end of which the polarity was reversed for about 45 seconds to remove any DNA stuck to the tubing. The electroeluate was transferred into a quartz spectrophotometer cuvette and the optical density read against the elution buffer. The concentration of the oligodeoxyribonucleotide was calculated from the optical density of the solution. The oligodeoxyribonucleotide was then concentrated by butanol extraction until the volume of the aqueous layer was about 500  $\mu$ l. A phenol chloroform 1:1 mixture (500  $\mu$ l) was then added, mixed and the mixture centrifuged at 13,500 r.p.m for 2 minutes. The aqueous phase was removed and concentrated further by butanol extraction to about 50  $\mu$ l. This was then dried under vacuum then reconstituted to 1pmole. $\mu$ l with autoclaved deionised water (Milli Q).

The purity of the oligodeoxyribonucleotide was checked by labelling 1pmole of the purified deoxynucleotide at the 5' terminal hydroxyl group using  $\gamma$ -<sup>32</sup>P ATP 100 $\mu$ Ci (2 pmoles) (Amersham) and T<sub>4</sub> polynucleotide kinase (4 units) in a 20  $\mu$ l reaction volume containing 50mM Tris HCl pH7.6, 10mM MgCl<sub>2</sub>, 5mM DTT, 100 $\mu$ M spermidine and 100 $\mu$ M EDTA. The kinase reaction was carried out at 37 °C for 20 minutes. Then 2 $\mu$ l of the reaction mix were added to 5 $\mu$ l of the formamide dye, boiled for 5 minutes put immediately on ice and run on a 15% denaturing polyacrylamide gel (7M Urea) prerun for 1 hour at 500V and electrophoresed for 3 hours at 800V. The gel was exposed to X-ray film for 30 minutes and the resulting autoradiograph showed that the gel purified oligodeoxynucleotide was of the required high purity.

## 2) Preparation of 1-[2'-Deoxy- $\beta$ -D-ribofuranosyl-5'-O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl-3'-(methoxy-N-morpholinophosphine)] naphthalene

1-[2'-Deoxy- $\beta$ -D-ribofuranosyl-5'-O- $\alpha,\alpha$ -bis(p-methoxyphenyl) benzyl]naphthalene (0.87g, 1.6mmole)

[prepared as described in 2a below] was stirred with chloromethoxy-N-morpholinophosphine (0.3g, 1.6mmole) and N, N'-diisopropylethylamine (0.85g, 6.6mmole) in CH<sub>2</sub>Cl<sub>2</sub> (30ml) at -40 °C under N<sub>2</sub>. The crude product was obtained after 10 min at ambient temperature by removal of the solvent under reduced pressure. The product 1-[β-D-2'-deoxyribofuranosyl-5'-O-α,α-bis(p-methoxyphenyl)benzyl-3'-(methoxy-N-morpholinophosphine)]naphthalene (0.8g, 73%) was isolated as a white solid by flash chromatography on silica gel (Merck Art 9385) with eluant of EtOAc: Et<sub>3</sub>N (66:1).

M/S (M + H)<sup>+</sup> 694 δ (CDCl<sub>3</sub>, 200MHz); 7.83 (3H,m,ArH), 7.33 (13H,m,ArH), 6.82(4H,m,ArH), 5.88 (1H,dd,H-1'), 4.65 and 4.48 (1H,m,H-3'), 4.3 and 4.15 (1H,m,H-4'), 3.8 (7H,m,OCH<sub>3</sub> and H-5'), 3.6 and 3.52 (4H,2xt morpholino protons), 3.46 and 3.39 (3H, 2xd,POCH<sub>3</sub>), 3.12 and 3.03 (4H,m,Morpholino protons), 2.57 (1H,m,H-2'), 2.18 (1H,m,H-2').

a) Preparation of 1-[2'-Deoxy-β-ribofuranosyl-5'-O-α,α-bis (p-methoxyphenyl)benzyl]naphthalene.

1-(2'-Deoxy-β-D-ribofuranosyl)naphthalene [(0.88g, 4 mmole) prepared as described in 2b below] was dried by co-evaporations with anhydrous pyridine (2 x 10ml) and then stirred at ambient temperature for 2h with α,α-bis(p-methoxyphenyl)benzyl chloride (1.3g, 4.3mmole) in anhydrous pyridine (10ml). Methanol (5ml) was added and the residual oil remaining after the solvent was removed in vacuo taken up in chloroform (50ml). The crude product was obtained by successively washing the organic solution with equal volumes of a saturated solution of sodium hydrogen carbonate (2x) and with water (2x) and then removal of the solvent under reduced pressure after drying over magnesium sulphate (MgSO<sub>4</sub>). The product 1-[2'-deoxy-β-D-ribofuranosyl-5'-O-α,α-bis(p-methoxyphenyl)benzyl] naphthalene (0.6g; 30.5%) was isolated as a pale brown solid by chromatography on silica gel (Merck Art 9385) with elution by pyridine: methanol: dichloromethane (1:3:196), followed by co-evaporation with toluene.

δ(CDCl<sub>3</sub>, 200MHz); 8.03 to 6.82, (20H,m, ArH), 5.88 (1H, m, H-1'), 4.47 (1H, m, H-3'), 4.12 (1H, m, H-4'), 3.78 (7H, m, 2x OCH<sub>3</sub> and H-5'), 3.43 (1H, m, H-5'), 2.50 (1H, m, H-2'), 2.12 (1H, m, H-2').

b) Preparation of 1-(2'-Deoxy-β-D-ribofuranosyl)naphthalene

1-[2'-deoxy-β-D-ribofuranosyl-3',5'-(1,1,3,3 tetraisopropylidisiloxy)] naphthalene [(0.12g, 0.24m mole) prepared as described in 2c below] in a mixture of pyridine (10%); water (10%) and tetrahydrofuran (80%), (2ml) was added to a solution of n-tetrabutylammonium fluoride (0.73m mole) in THF and allowed to stand at ambient temperature for 16 hours. The product, 1-(2'-deoxy-β-D-ribofuranosyl) naphthalene (19mg, 35%) was isolated as a white solid after removal of the solvent in vacuo and purification by chromatography on silica gel (Merck Art 9385) with elution by methanol:dichloromethane (1:9).

δ(CD<sub>3</sub>OD, 200MHz); 8.05(1H,m,ArH), 7.80(3H,m,ArH) 7.48(3H,m,ArH), 5.87(1H,dd,H-1'), 4.35(1H,ddd,H-3'), 4.05 (1H,ddd,H-4'), 3.75(2H,dd,2H-5'), 2.48(1H,ddd,H-2'), 1.99 (1H,ddd,H-2')

c) Preparation of 1-[2'-Deoxy-β-ribofuranosyl -3',5'-(1,1,3,3-te tetraisopropylidisiloxy)]naphthalene.

1-[β-D-Ribofuranosyl-2'-phenylthioformate-3',5'-(1,1,3,3-traisopropylidisiloxy)]naphthalene. [(0.4g, 0.6mmole) prepared as described in 2d below] and 2,2'-azobis(2-methylpropionitrile), (0.1g, 0.6mmole) and n-tributyltin hydride (2.8g, 2.7mmole) were refluxed in toluene (20ml) for 30 minutes. The product, 1-[β-D-2'-deoxyribofuranosyl-3'-5'-(1,1,3,3-tetraisopropylidisiloxy)]naphthalene (0.12g, 51%) was isolated by removal of the solvent under reduced pressure and purification on silica gel (Merck Art 9385) with elution by petrol (b.p.40/60): dichloromethane (1:1.5). M.S M (486).

d) Preparation of 1-[β-D-Ribofuranosyl-2'-phenylthioformate-3',5' (1,1,3,3-tetraisopropylidisiloxy)]-naphthalene

1-[β-D-Ribofuranosyl-3',5'-(1,1,3,3-tetraisopropylidisiloxy)]naphthalene [(0.4g, 0.8mmole) prepared as described in 2e below] in dry acetonitrile (25ml) was stirred for 24 hours at ambient temperature with 4-dimethylaminopyridine (0.6g, 4.8mmole) and phenylthiochloroformate (0.7g, 4.0mmole). Ethyl acetate (200ml) was added and the solution sequentially washed with equal volumes of a saturated solution of sodium hydrogen carbonate and with water. The crude product was obtained after drying the solution over

magnesium sulphate and removing the solvent under reduced pressure. The product 1- $\beta$ -D-ribofuranosyl-2'-phenylthioformate-3',5'-(1,1,3,3-tetraisopropylidisiloxy)]naphthalene (0.45g, 88%) was isolated as a white solid by flash chromatography on silica gel (Merck Art 9385) with elution by ethyl acetate: petrol (b.p.40/60) (1:12.3). M/S (M + NH<sub>4</sub>)<sup>+</sup> 656, (M + H)<sup>+</sup> 639.

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#### e) Preparation of 1- $\beta$ -D-Ribofuranosyl-3'-5'-(1,1,3,3-tetraisopropylidisiloxy)]naphthalene

1- $\beta$ -D-Ribofuranosyl)naphthalene [(1.0g, 3.8mmole) prepared as described by Ohnui,H.; Kuzuhara,H.; Emoto,S. Agr. Biol. Chem. 1972, 36 (9), 1651] in dry pyridine (15ml) was stirred for 2 hours at room temperature with 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane (1.6g, 5mmole). Dichloromethane (450ml) was added and the solution washed sequentially with equal volumes of a saturated solution of sodium hydrogen carbonate and saturated brine. The crude product, (2.6g) was obtained after drying the organic solution over magnesium sulphate and removal of the solvent under reduced pressure. The product, 1- $\beta$ -Ribofuranosyl-3',5'-(1,1,3,3-tetraisopropylidisiloxy)] naphthalene (0.8g, 1.6 mmole, 42%) was isolated as a colourless oil by flash chromatography on silica gel (Merck Art 9385) eluted with dichloromethane.

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M/S 520 (M + NH<sub>4</sub>)<sup>+</sup>, 503(M + H)<sup>+</sup>,  $\delta$ (CDCl<sub>3</sub>, 200 MHz); 8.12, 7.82, 7.52, (7H,m,ArH), 5.68 (1H,s,H-1'), 4.38 (1H,dd,H-3'), 4.28 (1H,d,H-5'), 4.18 (1H,d,H-2'), 4.13 (2H,m,H-4' and H-5'), 1.0 (28H,m,4 x CH(CH<sub>3</sub>)<sub>2</sub>)

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#### B) Preparation of 3'-deoxynaphthalene oligonucleotides (3) and (4)

##### 1) Oligonucleotide preparation

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The fully protected oligodeoxyribonucleotides of sequence (3) and (4) as hereinbefore defined were prepared as described in Preparation A1 above, except that the position 5 on an Applied Biosystems DNA synthesiser contained a solution of 1-[3'-deoxy-5'-O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl-2'-(methoxy-N,N-diisopropylaminophosphine)- $\beta$ -D-ribofuranosyl]naphthalene [(70.0mg) prepared as described in B2 below] in anhydrous CH<sub>3</sub>CN (1ml) and the phosphoramidite was coupled by a standard coupling reaction used for normal phosphoramidites.

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The oligodeoxyribonucleotide was cleaved from the solid support, deprotected and purified by the procedure described in Preparation A1 above.

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#### 2) Preparation of 1-[3'-deoxy-5'-O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl-2'-(methoxy-N,N-diisopropylaminophosphine)- $\beta$ -D-ribofuranosyl] naphthalene.

1-[3'-deoxy-5'-O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl- $\beta$ -D-ribofuranosyl]naphthalene (0.78, 1.3mmole) [prepared as described in 2a below] and N,N-diisopropylethylamine (0.67g, 5.2mmole) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (30ml) at -40°C was stirred with chloro-N-diisopropylmethoxyphosphine (0.26g, 1.3mmole). The reaction mixture was maintained under an atmosphere of dry N<sub>2</sub> and attained ambient temperature after 1 hour. The product, 1-[3'-deoxy-5'-O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl-2'-(methoxy-N,N-diisopropylaminophosphine)- $\beta$ -D-ribofuranosyl]naphthalene (0.8g, 87%) was isolated as a yellow solid (mixture of diastereoisomers) by removing the solvent in vacuo followed by flash chromatography on silica gel (Merck Art No. 9385) with elution by Et<sub>3</sub>N:EtOAc (1:66) and by evaporation of the solvent.

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$\delta$  (CDCl<sub>3</sub>, 200 MHz); 8.3 and 8.17 (1H,m,naphthalene protons), 7.80 (3H,m,naphthalene protons), 7.53 (3H,m,naphthalene protons), 7.28 (9H,m,ArH), 6.83 (4H,m,ArH), 5.8 and 5.75 (1H,s,H-1'), 4.65 (1H,m,H-4'), 4.5(18,m,H-2'), 3.78 (6H,s, 2x OCH<sub>3</sub>), 3.65 (2H,m,2H-5'), 3.45 and 3.36 (3H,d,POCH<sub>3</sub>), 2.09 and 2.02 (2H,m,2H-3'), 1.21 (14H,m,2CH(CH<sub>3</sub>)<sub>2</sub>).

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#### a) Preparation of 1-[3'-Deoxy-5'-O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl- $\beta$ -D-ribofuranosyl]naphthalene

1-(3'-Deoxy- $\beta$ -D-ribofuranosyl)naphthalene [(0.44g, 1.8mmole) prepared as described in 2b below] in dry pyridine (10ml) was treated with O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl chloride (0.97g, 2.9mmole) for 2 hours at ambient temperature. Excess O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl chloride was destroyed by the addition of MeOH (10ml) and CH<sub>2</sub>Cl<sub>2</sub> (50ml) was added. The crude product was obtained by successively

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washing the organic solution with  $\text{NaHCO}_3$  aq (2x50ml) and with water (2x50ml) and by evaporation of the solvent in vacuo after drying over  $\text{MgSO}_4$ . 1-[3'-Deoxy-5'-O- $\alpha$ , $\alpha$ -bis(p-methoxyphenyl)benzyl- $\beta$ -D-ribofuranosyl]naphthalene (0.7g, 71%) was isolated as a brown foam after flash chromatography on silica gel (Merck, Art 9385) with eluant of  $\text{MeOH}:\text{CH}_2\text{Cl}_2$  (1:99) and by removal of the solvent in vacuo followed

5 by co-evaporation with toluene.

$\delta(\text{CD}_3\text{OD}, 200\text{MHz})$ ; 8.18 (1H,m,naphthalene protons), 7.83 (3H,m,naphthalene protons), 7.5 (3H,m,naphthalene protons), 7.24 and 6.86 (13H,m,ArH), 5.6 (1H,br.s,H-1'), 4.62 (1H,m,H-4'), 4.37 (1H,m,H-2') 3.78(6H,d,  $\text{OCH}_3$ ), 3.43 (2H,dd,2H-5'), 2.07 (1H,ddd,H-3'), 1.86 (1H,ddd,H-3').

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#### b) Preparation of 1-(3'-Deoxy- $\beta$ -D-ribofuranosyl)naphthalene

1-(2',3'-Anhydro- $\beta$ -D-ribofuranosyl)naphthalene [(2.5g, 10.3mmole) prepared as described in 2c below] in dry THF (50ml) was added gradually to a stirred suspension of  $\text{LiAlH}_4$  (1.52g, 40mmole) in THF (50ml), then maintained in an atmosphere of  $\text{N}_2$  for 16 hours at ambient temperature. Excess  $\text{LiAlH}_4$  was destroyed by the gradual addition of  $\text{THF}/\text{H}_2\text{O}$  (95:5, 50ml) and the  $\text{Li}/\text{Al}$  complex decomposed with dilute  $\text{HCl}$  (1M, 50ml). The crude product was obtained by the addition of butan-1-ol (300ml) and by successively washing the organic solution with  $\text{NaHCO}_3$  aq (2x150ml) and water (2x150ml) followed by evaporation under reduced pressure. The product, 1-(3'-deoxy- $\beta$ -D-ribofuranosyl)naphthalene (0.44g, 17%) was isolated as a white crystalline solid after chromatography on silica gel (Merck, Art No. 9385) with eluant of  $\text{MeOH}:\text{CH}_2\text{Cl}_2$  (1:9) and evaporation of the solvent in vacuo.

$\delta(\text{CD}_3\text{OD}, 200\text{MHz})$ ; 8.18 (1H,m,ArH), 7.82 (3H,m,ArH), 7.5 (3H,m,ArH), 5.54 (1H,d,H-1'), 4.48 (1H,m,H-4'), 4.52 (1H,m,H-2'), 3.88 (2H,dd,2H-5') 1.93 (2H,m,2H-3'). M/S ( $\text{M} + \text{NH}_4$ )<sup>+</sup> 262. ( $\text{M} + \text{H}$ )<sup>+</sup> 244. mp. 145-147°C.

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#### c) Preparation of 1(2',3'-Anhydro- $\beta$ -D-ribofuranosyl)naphthalene

1-( $\beta$ -D-ribofuranosyl)naphthalene [(5.0g, 19.2mmole) prepared as described by Ohnui, H.; Kuzuhara, H.; Emote, S. Agr.Biol.Chem 1972, 36 (9), 1651] in acetonitrile (50ml) was treated with acetonitrile: water (99:1) (5ml) and 2-acetoxyisobutyl chloride (13.0g, 80mmole) and heated at 80°C for 1 hour. The reaction mixture was concentrated under reduced pressure and  $\text{EtOAc}$  (200ml) added. The solution was sequentially washed with saturated  $\text{NaHCO}_3$  aq (3x200ml), and  $\text{H}_2\text{O}$  (3x200ml) and concentrated under reduced pressure after drying over  $\text{MgSO}_4$ . The resulting brown oil was taken up in  $\text{MeOH}$  (30ml) and treated with sodium methoxide (2M, 30ml) at ambient temperature for 16 hours. The crude product was obtained by stirring with Amberlite IRC 50 (5g, 50mg) for 16 hours and by removing the solvent in vacuo. The product 1-(2',3'-anhydro- $\beta$ -D-ribofuranosyl)naphthalene (2.5g, 54%), was isolated as a white solid by chromatography on silica gel (Merck Art No 9385) with elution by  $\text{Et}_3\text{N}:\text{MeOH}:\text{CH}_2\text{Cl}_2$  (1:10:189)

$\delta((\text{CD}_3)_2\text{SO}, 200\text{MHz})$ ; 8.12, 7.93 and 7.58 (7H,m,ArH), 5.77 (1H,s,H-1'), 4.94 (1H,t,OH), 4.16 (1H,dd,H-4'), 4.13 (1H,d,H-2' or H-3'), 3.99 (1H,d,H-3' or H-2'), 3.5 (2H,m,2H-5').

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#### C) Preparation of C<sub>16</sub>-alkylene oligonucleotide (6)

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##### 1) Oligonucleotide preparation

The fully protected oligodeoxyribonucleotide (6) was prepared as described in Preparation A1 except that position 5 on the Applied Biosystems DNA synthesiser contained a solution of N,N'-diisopropylamino-[16-O- $\alpha$ , $\alpha$ -bis(p-methoxyphenyl)benzyl]hexadecan-1-O] methoxyphosphine [72mg prepared as described in 1a below] in acetonitrile: 1,2-dichloroethane (3:2) (1ml) and the C-16 phosphoramidite was coupled by a standard coupling reaction used for normal phosphoramidites. The oligodeoxyribonucleotide was cleaved from the solid support, and 2ml of concentrated ammonia added. Deprotection was carried out overnight at 55°C in a waterbath. The oligonucleotide was dried under vacuum and resuspended in 1ml of autoclaved deionised water (Milli Q) and stored at -20°C ready for purification. 100 $\mu$ l of the deprotected oligonucleotide was applied to an anion exchange column (Mono Q, Pharmacia) equilibrated with Buffer A (10mM NaOH, 0.5M NaCl). Flow rate was set to 1ml/min, chart speed 5mm/min, Absorbance Scale 1.0 Fraction size 0.5ml. The oligonucleotide was eluted with a gradient of Buffer B (10mM NaOH, 0.9M NaCl) O-

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100% in 60 minutes.

A number of small peaks eluted as the gradient increased followed by two larger peaks. Peak 1 corresponds approximately to a 30mer (or less) whereas peak 2 corresponds to about a 60 mer. It is therefore probable that peak 2 contains the full length product. Fractions corresponding to each of the peaks were pooled (for each peak), separately concentrated by drying under vacuum and desalted, on a desalting and buffer exchange column (PD10, Pharmacia) ready for use.

Confirmation of the identity of the correct peak was determined by checking the purity of the oligodeoxyribonucleotides as described in Preparation A1, except that both peaks of oligonucleotide 6 were labelled. Autoradiography reveals that peak 2 represents the required oligodeoxyribonucleotide.

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a) Preparation of N,N-Diisopropylamino-[16-O- $\alpha,\alpha$ -bis (p-methoxyphenyl)benzyl] hexadecan-1-O]-methoxyphosphine

15 16-O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzylhexadecan-1-ol prepared as described in 1b below] in  $\text{CH}_2\text{Cl}_2$  (15ml) was stirred with chloro(methoxy)-N -diisopropylphosphine (0.36g, 1.79mmole) in the presence of N , N -diisopropylethylamine (0.92g, 7.2mmole) at ambient temperature for 2 hours. The product, N -diisopropyl [16-O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl hexadecan-1-O -] methoxyphosphine, (0.62g, 48%) was isolated as a yellow oil by removing the solvent in vacuo and by chromatography on silica gel (Merck Art 9385) with eluant of  $\text{Et}_3\text{N}$ :hexane (1:9).

20 M/S 722 (M+H)<sup>+</sup>,  $\delta$  ( $\text{CDCl}_3$ , 200MHz): 7.3 and 6.8 (13H,m,Ar), 3.80 (6H,s, $\text{OCH}_3$ ), 3.59 (2H,m  $\text{CH}_2\text{OP}$ ), 3.4 (3H,d, $\text{POCH}_3$ ), 3.03 (2H,t, $\text{CH}_2\text{ODMT}$ ), 1.25 (42H,m,14C  $\text{H}_2$ , 2C  $\text{H}$  (C  $\text{H}_3$ )<sub>2</sub>).

25 b) Preparation of 16-O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl] hexadecan-1-ol

1,16-Hexadecanediol (1.0g, 3.8mmole) was treated with  $\alpha,\alpha$ -bis(p-methoxyphenyl)benzyl chloride (1.28g, 3.8mmole) in pyridine (30ml) at ambient temperature for 16 hours. The solvent was removed in vacuo and the product, 16-O- $\alpha,\alpha$ -bis(p-methoxyphenyl)benzylhexadecan-1-ol, (0.35g, 16%) was isolated as a brown oil by flash chromatography on silica gel (Merck Art 9385) with eluant of  $\text{MeOH}:\text{CH}_2\text{Cl}_2$  (1:99). M/S (M+H)<sup>+</sup> 561

D) Preparation of oligonucleotide (8)

35 The fully protected oligodeoxyribonucleotide (8) was prepared as described in Preparation A1 above, except that position 5 on the Applied Biosystems DNA synthesiser contained a solution of the N , N -diisopropylaminomethylphosphoramidite of 5'-dimethoxytrityl-2'-deoxythymidine (0.25g) in anhydrous acetonitrile (3.8ml) and the methylphosphoramidite was coupled by a standard coupling reaction used for normal phosphoramidites. The procedures consisted briefly of: (1) removal of the  $\alpha,\alpha$ -bis(p-methoxyphenyl)-benzyl group with 3% trichloroacetic acid in dichloromethane; (2) coupling of N , N -diisopropylaminomethylphosphoramidite of 5'-dimethoxytrityl-2'-deoxythymidine activated by tetrazole for 30 sec; (3) iodine oxidation of the intermediate phosphite to a phosphate; and (4) a capping step with acetic anhydride.

45 Alternatively, the fully protected oligodeoxyribonucleotide sequence may be prepared by the manual methods as described by Atkinson and Smith in 'Oligonucleotide Synthesis, a Practical Approach' (M.J. Gait Editor, IRL Press, Oxford, Washington DC, pages 35-81). The detritylated oligodeoxyribonucleotide sequence was cleaved from the solid support and deprotected by treatment with ethylene diamine: ethanol (1:7 0.4ml) at 55°C for 55 minutes. The ethylene diamine in ethanol solution was evaporated at 50°C under reduced pressure and the residue was treated again with the ethylene diamine:ethanol solution at 55°C for 55 minutes. After a second evaporation under reduced pressure the residue was dissolved in water (1ml). An aliquot of the solution containing the oligodeoxyribonucleotide was purified by polyacrylamide gel electrophoresis as described in Preparation A1.

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Example 2

This example demonstrates the ability of as little as one modified base to prevent polynucleotide tail

amplification. The following oligonucleotides were prepared using methods analogous to those outlined in Example 1 above:-

Oligonucleotide 2a;

AATTCGTCGATAAGGCTGTGCTGNTCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG (where N is as described in oligonucleotide 2 in Example 1)

Oligonucleotide 4a;

AATTCGTCGATAAGGCTGTGCTGNTCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG (where N is as described in oligonucleotide 3 in Example 1).

Oligonucleotide 8a;

AATTCGTCGATAAGGCTGTGCTGNTCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG (where N is as described in oligonucleotide 8 in Example 1).

The following reactions were then effected using procedures identical to those disclosed in Example 1:-

				Length of product expected if naphthalene bridge stops enzyme
15	a1)	Labelled 5		30 (No template/ no dNTPs)
20	a2)	" 5		30 " "
	a3)	" 5	+ dNTPs (ACGT)	30 (No template/ no extension)
	b)	" 5	+ 1	30 (No dNTPs)
25	c)	" 5	+ 2a	30 (No dNTPs)
	d)	" 5	+ 4a	30 (No dNTPs)
	e)	" 5	+ 8a	30 (No dNTPs)
	f)	" 5	+ 1 + dNTPs(ACGT)	63 Control
30	g)	" 5	+ 2a + dNTPs(AGCT)	35
	h)	" 5	+ 4a + dNTPs(AGCT)	35
	i)	" 5	+ 8a + dNTPs(AGCT)	35

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The results are shown in Figure 2 and demonstrate that a single 3' deoxynaphthalene residue will stop extension by Taq DNA polymerase, a single 2' deoxynaphthalene will partially stop extension by Taq DNA polymerase and a single 4-methyl phosphonate substituted thymidine residue is ineffective in stopping extension by Taq DNA polymerase.

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### Example 3

This example demonstrates the use of metaphosphate and thymidine phosphoramidate polymerisation blocking moieties to prevent polynucleotide tail amplification.

The following oligonucleotides were prepared:-

Oligonucleotide 20;

AATTCGTCGATAAGGCTGTGCTGNNNTCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG wherein N represents a thymidine phosphormorpholidate group

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Oligonucleotide 21;

AATTCGTCGATAAGGCTGTGCTGNTCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG wherein N represents a thymidine phosphormorpholidate group

Oligonucleotide 22;

AATTCGTCGATAAGGCTGTGCTGNTCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG wherein N represents a metaphosphate group

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Oligonucleotide 23;

AATTCGTCGATAAGGCTGTGCTGNNNTCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG wherein N represents a metaphosphate group

Preparation of metaphosphate linked oligonucleotide sequences

(a) The fully protected oligonucleotide of sequence:

5' AATTCCGTGCATAAGGCTGTGCTG 5 TCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG 3'

was prepared as described previously on an Applied Biosystems 380 A DNA Synthesiser, except that position 5 contained a solution of N,N-diisopropylamino-O-cyanoethyl-O-tritylmercaptamoethylphosphine (52 mgs) (Sinha N.D, Biarnot J., McManus J., and Koster A., Nucleic Acids Research, 1984, 12, 4539-4557) in anhydrous acetonitrile (1ml). The phosphoramidite at position 5 was coupled to the oligonucleotide bound to a controlled pore glass support by the standard procedures used for coupling normal phosphoramidites except that the trityl protecting group was removed by manual treatment with an aqueous solution of silver nitrate (0.25M, 1ml) at ambient temperature for 16 hours and, after washing with distilled water (2 x 1ml), by treatment with an aqueous solution of dithiothreitol (0.3M, 1ml, PH8.3) at ambient temperature for 16 hours. After washing the supported oligonucleotide sequence with distilled water (2 x 1ml) followed by drying under reduced pressure at 50 °C (5 minutes), the synthesis of the fully protected oligonucleotide sequences completed using a standard cyanomethylphosphoramidite cycle on the DNA Synthesiser.

The detritylated oligonucleotide sequences were cleaved from the solid support and all the protecting groups removed by treatment with aqueous ammonia solution (sp.gr. 0.88, 55 °C for 16 hours). After removal of the ammonia solution under reduced pressure at 50 °C, the residue was dissolved in sterile water (1ml) and the metaphosphate bridged oligonucleotides isolated by polyacrylamide gel electrophoresis.

(b) The fully protected oligonucleotide of sequence:

5' AATTCCGTGCATAAGGCTGTGCTG 555 TCGACGAGAAAGGGACTGAAGCTGCTGGGGCCCATG 3'

was prepared as described in preparation (a) except that the phosphoramidite at position 5 (N,N-diisopropylamino-O-cyanoethyl-O-tritylmercaptamoethylphosphine) was used in three standard cyanoethylphosphoramidite cycles with manual removal of the trityl protecting group before each coupling reaction. The synthesis, cleavage from the solid support and the deprotection of the bridged oligonucleotide sequences was completed as described in preparation (a) and the trimetaphosphate bridged oligonucleotides were isolated by polyacrylamide gel electrophoresis.

30 Preparation of phosphormorpholidate linked oligonucleotide sequences

(c) The fully protected oligonucleotide of sequence:

5' AATTCCGTGCATAAGGCTGTGCTG 5 TCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG 3'

35 were prepared as described previously using a standard cyanoethylphosphoramidite cycle on an Applied Biosystems 380A DNA Synthesiser, except that position 5 contained a solution of 5'-dimethoxytrityl-3'-thymidine H-phosphonate (250 mg) in anhydrous acetonitrile: pyridine (1:1, 6ml) and H-phosphonate reagents and protocols (Oligonucleotide Synthesis with Hydrogen-Phosphonate Monomers, Applied Biosystems User Bulletin, No 44, November 20, 1987) were used to incorporate the reagent at position 5 into the oligonucleotide sequence. The procedures consisted briefly of: (1) removal of the dimethoxytrityl group with 7% dichloroacetic acid in dichloromethane (2) coupling of the 5'-dimethoxytrityl-3'-thymidine H-phosphonate activated with 1-adamantanecarbonyl chloride for 40 seconds (3) a capping step with isopropylphosphite and 1-adamantanecarbonyl chloride and (4) the manual oxidation of the H-phosphonate diester with a solution of freshly distilled morpholine (10%) in carbon tetrachloride for 5 minutes. After incorporation of the reagent at position 5 into the oligonucleotide sequence, the synthesis of the fully protected oligonucleotide sequences was completed using standard cyanoethylphosphoramidite reagents and a standard cycle on the DNA synthesiser.

45 The detritylated oligonucleotide sequences were cleaved from the controlled pore glass support and the protecting groups removed by treatment with aqueous ammonia solution (sp.gr. 0.88, 55 °C, 16 hours). After removal of the ammonia solution under reduced pressure at 50 °C, the residue was taken up in sterile water (1ml) and the phosphormorpholidate bridged oligonucleotides isolated by polyacrylamide gel electrophoresis.

(d) The fully protected oligonucleotide of sequence:

5' AATTCCGTGCATAAGGCTGTGCTG 5555 TCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG 3'

55 was prepared as described in preparation (c) except that the detritylation, coupling and capping steps with 5'-dimethoxytrityl-3'-thymidine H-phosphonate at position 5 on an Applied Biosystems 380A DNA Synthesiser were repeated a further three times before the manual oxidation of the four H-phosphonate linkages with morpholine in carbon tetrachloride (10% solution for 5 minutes). The synthesis of the oligonucleotide

sequences were completed using normal cyanoethylphosphoramidite reagents and synthesis cycle and the detritylated oligonucleotide sequences were cleaved from the controlled pore glass support and deprotected by standard treatment with ammonia solution (sp.gr. 0.88, 55°C, 16 hours). After removal of the ammonia solution (reduced pressure at 50°C) and the addition of sterile water (1ml), the tetraphosphormorpholidate bridged oligonucleotide was isolated by polyacrylamide gel electrophoresis.

Isolation :- An aliquot of the solutions containing the oligodeoxyribonucleotides was purified by polyacrylamide gel electrophoresis as described in preparation A1.

The following reactions were then effected using procedures identical to those disclosed in Example 1:-

10	Length of product expected if naphthalene bridge stops enzyme			
15	a)	Labelled 5	+ dNTPs	30 (No template/ no extension)
	b)	" 5	+ 1	30 (No dNTPs)
	c)	" 5	+ 631	30 (No dNTPs)
20	d)	" 5	+ 632	30 (No dNTPs)
	e)	" 5	+ 652	30 (No dNTPs)
	f)	" 5	+ 657	30 (No dNTPs)
25	g)	" 5	+ 1 + dNTPs(ACGT)	63 Control
	h)	" 5	+ 631 + dNTPs(AGCT)	35
	i)	" 5	+ 652 + dNTPs(AGCT)	35
30	j)	" 5	+ 632 + dNTPs(AGCT)	35
	k)	" 5	+ 657 + dNTPs(AGCT)	35

The results are shown in Figure 3 and demonstrate that one and three metaphosphate moieties significantly inhibit extension by Taq DNA polymerase beyond the bridge. Four thymidine phosphormorpholidate moieties show useful blocking activity but a single thymidine phosphormorpholidate moiety shows negligible blocking activity.

Without wishing to be bound by any theoretical considerations the polymerase blocking activities may be underestimated because of the presence of the 63 mer bands in the controls with dNTPs omitted. The presence of these bands may be due to a carry over of polynucleotide kinase activity during combination of the template oligonucleotides with <sup>32</sup>P phosphorylated oligonucleotide 5.

#### Example 4

This example demonstrates a microtitre plate test capture system to determine whether:-

- capture oligonucleotides are bound to a microtitre plate,
- there is nonspecific binding of the test capture oligonucleotide sequences to immobilised sequences other than their complement, or to the protein coating on the microtitre wells.
- an alkaline phosphatase, nitro blue tetrazolium 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (AP NBT BCIP) colour development system performs satisfactorily with the above capture oligonucleotides.

#### Capture oligonucleotides

Oligonucleotide 13: TTTTTTTTTTATCAACTTACTTGCCCTATA

Oligonucleotide 14: TTTTTTTTTTAGATCTTGGTTAACAATCGT

Oligonucleotide 15; TTTTTCGGTCCTCATAATACATACT

these are used as capture oligonucleotides for complementary hybridisation with oligonucleotides 11, 12 and 10 respectively as set out in Example 5 below. Oligonucleotide 13 is identical to oligonucleotide 7 as disclosed in Example 1. The 5' (dT)10 residues were included as spacer elements between the hybridisation regions and their respective conjugants.

These oligonucleotides were immobilised on protein coated microtitre plates as follows with reference to Figure 4:-

10	Oligonucleotide 15	A1 B1 C1 D1 E1 F1
	Oligonucleotide 14	B2 D2 F2
	Oligonucleotide 13	A2 C2 E2
	No oligonucleotide	A3 B3 C3 D3 E3 F3

15

The chosen concentration was such that 100% binding represents 1 nmole oligonucleotide per well.

## 20 Test capture oligonucleotides

Each oligonucleotide is a unique sequence, complementary to one of the capture oligonucleotides described above, and conjugated to the enzyme alkaline phosphatase (AP) using the E-link<sup>TM</sup> oligonucleotide labelling kit (Cambridge Research Biochemicals). The labelling was carried out with a nominal concentration of each oligonucleotide (1.5  $\mu$ M).

25 Oligonucleotide 16;

TTTTTTTTTTAGTATGTATTATGAGGACCG

Oligonucleotide 17;

TTTTTTTTTTATAGGCAAGTAAGTTGATA

30 Oligonucleotide 18;

TTTTTTTTTTACGATTGTTAACCAAGATCT

these are used as capture control oligonucleotides

## 35 Experimental details

200  $\mu$ l of prehybridisation solution, containing 0.6M NaCl, 20mM sodium phosphate pH 7.5, 1mM EDTA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin, was added to each well and incubated in the dark at 37°C minutes. This was then removed by pipetting. The appropriate oligonucleotide enzyme conjugate was diluted 1 in 10 in a polymerase chain reaction (PCR) mix (100  $\mu$ M dATP, 100 $\mu$ M dGTP, 100 $\mu$ M dCTP & 100 $\mu$ M dTTP), 1.2mM MgCl<sub>2</sub>, 50mM KCl, 10mM Tris HCl pH 8.3, 0.01 gelatine) and 10 $\mu$ l of diluted conjugate, ie. approximately 1.5 pmols were added to 40 $\mu$ l of 5X SSC (1.1 MNaCl, 0.825M sodium citrate pH 7.0 already added to each well). This was allowed to incubate in the dark at 37°C for 1 hour. Solutions were then removed by pipetting. Each of the test capture control oligonucleotides was added to a different set of microtitre wells as prepared above and with reference to Figure 4:-

50	Oligonucleotide 16	A1 A2 A3 B1 B2 B3
	Oligonucleotide 17	C1 C2 C3 D1 D2 D3
	Oligonucleotide 18	E1 E2 E3 F1 F2 F3

Following hybridisation, the wells were washed for 2 x 5 minutes at room temperature using 200 $\mu$ l of 2X SSC (0.44M NaCl 0.33M sodium citrate pH 7.0). Solutions were again removed by pipetting.

The final step was addition of 200 $\mu$ l of a colour development solution comprising alkaline phosphatase (AP) 9.5 buffer (0. 1M Tris HCl pH 9.5, 0.1M NaCl, 5mM MgCl<sub>2</sub>) and containing NBT (0.33mg/ml) and BCIP (0.17mg/ml). The colour development solution was prepared as follows:- for 15 ml of reagent, 5mg of NBT

was suspended in 1.5ml AP 9.5 buffer in a microcentrifuge tube, vortexed vigorously for 1-2 minutes and then centrifuged briefly. The supernatant was decanted into 10ml of AP 9.5 buffer, and warmed to 37° C in a polypropylene tube. The residual NBT pellet was extracted twice more with 1.5ml of AP 9.5 buffer and these supernatants pooled with the original solution. The tube was rinsed with a final 0.5ml of AP 9.5 buffer and also decanted into the 15 ml NBT stock solution. BCIP (2.5mg) was dissolved in 50µl of N,N-dimethylformamide and added dropwise with gentle mixing into the NBT solution. The solution was frozen at -20° C in 1 ml aliquots and then before use the solution was prewarmed to 37° C in a water bath. Colour was allowed to develop in the dark at 37° C and photographs were taken after 15 minutes and 2 hours 15 minutes.

The results are shown in Figure 4 and show colour development only in wells A1 B1 C2 and F2 which is entirely consistent with the test capture oligonucleotides hybridising only to their complementary immobilised oligonucleotides under the conditions employed.

### 15 Example 5

This example demonstrates the specific capture and detection of amplification products based on the S locus of the alpha-1-antitrypsin gene and using an internal amplification control based on sequence from exon V of the same gene. Detection of the immobilised products was achieved using an alkaline phosphatase (AP) conjugated common detection primer. This common primer was prepared using the E-link™ conjugation kit (Cambridge Research Biochemicals) as directed by the supplier and is complementary to the detection tail of all the amplified products.

The following oligonucleotides were used wherein N represents a 3'-deoxyribofuranosyl naphthalene moiety, the naphthalene moieties being linked via the 2'-PO<sub>4</sub> grouping to the adjacent nucleotide:-

25 Oligonucleotide 8b;

GTGTCGTCCCGCAGTCAATGNNNNCCCACCTTCCCCTCTCTCCAGGCAAATGGG

this oligonucleotide is the common signal primer for carrying out the ARMS assay method.

Oligonucleotide 9

GTCTCGTCCCGCAGTCAATGNNNNGAGACTTGGTATTTTGTTCATCATTAAAG

30 Oligonucleotide 10

AGTATGTATTATGAGGACCGNNNNGTCCACGTGAGCCTTGCTCGAGGCCTGGG

these oligonucleotides are for the exon V control reaction and are the signal and capture primers respectively.

Oligonucleotide 11;

35 TATAGGCAAGTAAGTTGATANNNTGGTGATGATATCGTGGGTGAGTTCATTTT

Oligonucleotide 12;

ACGATTGTAAACCAAGATCTNNNNTGGTGATGATATCGTGGGTGAGTTCATTTA

oligonucleotides 11 and 12 are both capture primers and comprise respectively regions for complementary hybridisation to the published normal and mutant sequences of the S locus of the alpha-1-antitrypsin gene.

40 Oligonucleotide 13;

TTTTTTTTTTTATCAACTTACTTGCCCTATA

Oligonucleotide 14;

TTTTTTTTTTTAGATCTTGGTTAACAATCGT

Oligonucleotide 15;

45 TTTTTTTTTTCGGTCCTCATAATACATACT

these are used as capture oligonucleotides for complementary hybridisation with oligonucleotides 11, 12 and 10 respectively. Oligonucleotide 13 is identical to oligonucleotide 7 as disclosed in Example 1. The 5'-(dT)<sub>10</sub> residues were included as spacer elements between the hybridisation regions and their respective conjugants.

50 Oligonucleotide 16;

TTTTTTTTTTTAGTATGTATTATGAGGACCG

Oligonucleotide 17;

TTTTTTTTTTTATAGGCAAGTAAGTTGATA

Oligonucleotide 18;

55 TTTTTTTTTTACGATTGTTAACCAAGATCT

these are used as capture control oligonucleotides

Oligonucleotide 19;

TTTTTTTTTTTCATTGACTGCGGGACGACAC

this is the common signal oligonucleotide for the ARMS assay method

#### Preparation of oligonucleotides

Oligonucleotides 13-15 and 16-19 were synthesised as 5' aminoalkyl derivatives using aminoalkyl phosphoramidite (Amino-link 2, Applied Biosystems) in the final synthesis cycle on the DNA synthesiser. These oligonucleotides were conjugated to alkaline phosphatase (AP) using the E-LINK™ oligonucleotide labelling kit as directed by the supplier (Cambridge Research Biochemicals).

#### Oligonucleotide purification

Aliquots of crude oligonucleotides 8b, 9, 10, 11 and 12 (225µl) were precipitated by the addition of 25µl of 3M sodium acetate pH 5.2, mixing and then adding 750µl ethanol. After further mixing the tubes were left at -20 °C for one hour and spun for 10 minutes at 13,500 rpm before removing the supernatant. The pellets were left to air dry for 20 minutes then reconstituted with 50µl formamide dye (80% formamide, 10mM NaOH, 1mM EDTA, 0.1% bromophenol blue). The samples were then loaded onto a 15% acrylamide gel in 7M urea, 100mM Tris borate EDTA buffer pH 8.3, which had been prerun for 1 hour at 300V. The samples were electrophoresed overnight at 140V, ie. until the dye had run to the bottom of the gel (0.3 x 24 x 17 cm<sup>3</sup>). The oligonucleotides were visualised by ultra violet shadowing at 254nm on a 20 x 20 aluminium backed thin layer chromatography plate (Merck 5554) covered with Saran wrap. The bands were excised using a new scalpel for each band to prevent cross-contamination. The gel slices were placed separately into a length of treated dialysis tubing (boiled in 1mM EDTA for ten minutes then rinsed 3X by boiling in milli Q water) containing 1ml 10mM Tris borate EDTA buffer pH 8.3. The oligonucleotides were electroeluted from the gel slice at 2100V for 1.5 hours, the polarity was then reversed for 45 seconds. The eluates were removed into 1.5ml quartz spectrophotometer cuvettes, the optical density at 220-330 nanometres was then scanned against the elution buffer and the concentration of oligonucleotide calculated based on the optical density at 260nm. The oligonucleotides were then concentrated by butanol extraction until the volume of the aqueous layer was ~500 µl. A phenol chloroform 1:1 (500µl) extraction was included. The aqueous phase was removed and further concentrated by butanol extraction to ~50µl. This was then dried under vacuum and reconstituted with filtered MilliQ water to 50 pmoles µl ready for the amplification reactions.

#### Thiolation of capture oligonucleotides 13, 14 & 15

The capture oligonucleotides (200µl, 0.2µM synthesis) were mixed separately with 4mg/ml iminothiolane in 0.2M Na<sub>2</sub>CO<sub>3</sub>/HCO<sub>3</sub> pH 9.6 (300µl) and incubated at ambient temperature for 1 hour. The reactions were diluted to 1ml with phosphate buffered saline (PBS) and desalted on a Nap-25 column (Pharmacia). The columns were washed with PBS (2.2ml). The first 1.6ml eluted was retained and the oligonucleotide concentrations were measured by u.v. spectrometry.

#### Immobilisation of thiolated oligonucleotides to microtitre dishes

Oligonucleotide immobilisation to microtitre wells was performed using a modification of the method described by Running, J.A. and Urdea, M.S., (1990), Biotechniques, 8, 276-277. Poly (Phe-Lys) (Sigma) was dissolved to 100µg/ml in 50mM Na<sub>2</sub>CO<sub>3</sub>/HCO<sub>3</sub>, pH 9.6 and 100µl of this solution was added to each well of five clear polystyrene microtitre dishes (Nunc). The dishes were incubated overnight at 4°C then washed 3 times with PBS/0.05% Tween. Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) was dissolved at 8.7mg/ml in dry dimethylformamide and this solution was diluted 10 times with conjugation buffer (100mM triethanolamine HCl, 1mM MgCl<sub>2</sub>, 1mM ZnSO<sub>4</sub>, pH 7.4). 100µl of this solution was added to each microtitre well and the plates were incubated at ambient temperature for 1 hour. The wells were washed 5 times with water. Thiolated oligonucleotides, 10µM in PBS, were added to appropriate microtitre wells at a rate of 100µl per well. These were incubated overnight at 4°C. The wells were washed twice with PBS/0.05% Tween, twice with 5% lactose, 0.5% gelatin, 0.1% N<sub>3</sub>Na, 6mM PBS, pH 7.5. Finally the coated dishes were dried overnight in a laminar flow cabinet. The dishes were stored at 4°C in vacuo.

Generation of PCR products with non amplifiable tails

ARMS analysis of a homozygous S variant of the alpha-1-antitrypsin gene:-

5        1 µl DNA was added to a tube containing 1.2mM MgCl<sub>2</sub>, 50mM KCl, 10mM Tris HCl pH 8.3, 0.01% gelatin, and 100 µM dATP, 100µM dGTP, 100µM dCTP & 100µM dTTP to make a reaction volume of 98 µl to a final concentration of 100 µM each and 50 p moles each of oligonucleotides 8b, 9, 10 and 11 (the ARMS primer specific for the normal allele). 1 µl DNA was also added to a similar tube containing  
10        oligonucleotide 12 (the ARMS primer specific for the S variant allele) instead of oligonucleotide 11. 2 drops of mineral oil were added to each tube and spun for 1 minute at 13500 rpm. The tubes were transferred into a PCR machine (Techne) where they were denatured at 95 °C for 5 minutes before 2 units of Taq polymerase (Perkin Elmer, Ampli-Taq) were added still at 95 °C to make the final volume 100 µl. The Techne machine was switched into the 2 step PCR programme of 2 minutes at 94 °C, 4 minutes at 60 °C,  
15        35 cycles with a final 9.9 minutes extension step at 60°C. A 20 µl aliquot of the reaction mixtures was electrophoresed on 1.5% agarose gel. This was to ascertain the genotype of the DNA sample by traditional analytical methods.

The results are shown in Figure 6:-

Lane 4 S normal allele specific ARMS primer

20        Lane 5 S variant allele specific ARMS primer

The control (lower band) is present in both lanes whereas there is only a product band in lane 5 (S variant). This confirms that the DNA is from a homozygote for the S variant allele of the alpha-1-antitrypsin gene.

25        Solid phase capture and detection of ARMS products

Oligonucleotide 19, the common signal primer was conjugated to alkaline phosphatase using the E-link<sup>TM</sup> oligonucleotide labelling kit (Cambridge Research Biochemicals). The solutions are the same as those described in detail in Example 4 above unless otherwise stated:-

- 30        1. 200µl prehybridisation solution was added to each well. This was then incubated at 37 °C with shaking for 30 minutes. The solution was removed by pipetting.
2. A 20µl aliquot of PCR product from the S normal primer tube was added to wells [A1, A2 and A3 with reference to Figure 7] containing 60µl 5X SSC. A 20 µl aliquot of PCR product from the S variant primer tube was added to wells [B1, B2 and B3 with reference to Figure 7] also containing 60 µl 5 x SSC.
- 35        Hybridisation lasted for 1 hour at 37 °C with shaking. The solutions were then removed by pipetting. Signal generation was as described in Example 4.
3. 10µl diluted conjugated oligonucleotide 19 ie. ~1.5 pmoles was added to 40µl 5X SSC already added to each well. Hybridisation lasted for 1 hour at 37 °C with shaking. The solutions were removed by pipetting.
- 40        4. 2x 5 minutes washes with 200µl 2 X SSC at 37 °C were carried out with shaking. The solutions were removed by pipetting.
5. 200µl of colour development solution was added to each well [A1, A2, A3, B1, B2 and B3 with reference to Figure 7] and these were incubated for ~1 hour at 37 °C with shaking. The wells were then scanned using a Hewlett Packard Scan Jet Plus scanner and digitised using Apple Mackintosh software.

45        The results are shown in Figure 7. It can be seen that both the internal control wells A1 and B1 are coloured. In addition only the variant well B2 is coloured providing a clear diagnosis that the DNA is from a homozygous S variant of alpha-1-antitrypsin. There is no significant nonspecific binding, ie. there is no colour in the control wells A3 and B3.

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The sequence identities 1-23 set out on the following pages correspond respectively to oligonucleotides 1-23 as hereinbefore described:-

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(1) GENERAL INFORMATION

10

(i) APPLICANT: Imperial Chemical Industries PLC

(ii) TITLE OF INVENTION: AMPLIFICATION PROCESSES

15

(iii) NUMBER OF SEQUENCES: 27

(iv) CORRESPONDENCE ADDRESS:

20

(A) ADDRESSEE: Legal Department: Patents

(B) STREET: Bessemer Road

(C) CITY: Welwyn Garden City

(D) STATE: Hertfordshire

25

(E) COUNTRY: United Kingdom

(F) ZIP: GB-AL7 1HD

30

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 5.25 inch, 1.2 Mb storage

(B) COMPUTER: Tandon

(C) OPERATING SYSTEM: PC-DOS 3.20

35

(D) SOFTWARE: ASCII from WPS-PLUS

(vi) CURRENT APPLICATION DATA:

40

(A) APPLICATION NUMBER

(B) FILING DATE:

45

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBERS: GB 8920097.6

(B) FILING DATE: 06-SEP-1989

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55

SEQ ID No 1

SEQUENCE LENGTH: 63

5 SEQUENCE TYPE: Nucleotide  
STRANDEDNESS: Single  
TOPOLOGY: Linear

10 AATTCCTGTC ATAAGGCTGT GCTGACCATC GACGAGAAAG GGAAGTGAAGC TGCTGGGGCC 60  
ATG 63

15 SEQ ID No 2

SEQUENCE LENGTH: 63

20 SEQUENCE TYPE: Nucleotide  
STRANDEDNESS: Single  
TOPOLOGY: Linear

N: 2'-deoxyribofuranosyl naphthalene  
25 AATTCCTGTC ATAAGGCTGT GCTGNNNTTC GACGAGAAAG GGAAGTGAAGC TGCTGGGGCC 60  
ATG 63

30 SEQ ID No 2a

SEQUENCE LENGTH: 60

35 SEQUENCE TYPE: Nucleotide  
STRANDEDNESS: Single  
TOPOLOGY: Linear

N: 2'-deoxyribofuranosyl naphthalene  
40 AATTCCTGTC ATAAGGCTGT GCTGNTTCGAC GAGAAAGGGA CTGAAGCTGC TGGGGCCATG 60

45 SEQ ID No 3

SEQUENCE LENGTH: 61

50 SEQUENCE TYPE: Nucleotide  
STRANDEDNESS: Single  
TOPOLOGY: Linear

N: 3'-deoxyribofuranosyl naphthalene  
AATTCCTGTC ATAAGGCTGT GCTGNNTTCGAC GAGAAAGGGA ACTGAAGCTGC CTGGGGCCAT 60  
55 G 61

## SEQ ID No 4

5 SEQUENCE LENGTH: 63  
 SEQUENCE TYPE: Nucleotide  
 STRANDEDNESS: Single  
 10 TOPOLOGY: Linear  
 N: 3'-deoxyribofuranosyl naphthalene  
 AATTCCGTGC ATAAGGCTGT GCTGNNNNTC GACGAGAAAG GGACTGAAGC TGCTGGGGCC 60  
 15 ATG 63

## SEQ ID No 4a

20 SEQUENCE LENGTH: 60  
 SEQUENCE TYPE: Nucleotide  
 STRANDEDNESS: Single  
 25 TOPOLOGY: Linear  
 N: 3'-deoxyribofuranosyl  
 AATTCCGTGC ATAAGGCTGT GCTGNTCGAC GAGAAAGGGA CTGAAGCTGC TGGGGCCATG 60  
 30

## SEQ ID No 5

35 SEQUENCE LENGTH: 30  
 SEQUENCE TYPE: Nucleotide  
 STRANDEDNESS: Single  
 TOPOLOGY: Linear  
 40 CATGGCCCCAGCAGCTTCAGTCCCTTTCTC 30

## SEQ ID No 6

45 SEQUENCE LENGTH: 60  
 SEQUENCE TYPE: Nucleotide  
 STRANDEDNESS: Single  
 50 TOPOLOGY: Linear  
 N: C4 straight chain alkylene  
 AATTCCGTGC ATAAGGCTGT GCTGNNNNTC GACGAGAAAG GGACTGAAGC TGCTGGGGCC 60  
 55 ATG 63

## SEQ ID No 7

SEQUENCE LENGTH: 30

SEQUENCE TYPE: Nucleotide

STRANDEDNESS: Single

TOPOLOGY: Linear

TTTTTTTTTT TATCAACTTA CTTGCCTATA

30

## SEQ ID No 8

SEQUENCE LENGTH: 63

SEQUENCE TYPE: Nucleotide

STRANDEDNESS: Single

TOPOLOGY: Linear

N: thymidine 4-methyl phosphonate

AATTCCGTGC ATAAGGCTGT GCTGNNNNTC GACGAGAAAG GGAAGTGAAGC TGCTGGGGCC 60

ATG

63

## SEQ ID No 8a

SEQUENCE LENGTH: 60

SEQUENCE TYPE: Nucleotide

STRANDEDNESS: Single

TOPOLOGY: Linear

N: thymidine 4-methyl phosphonate

AATTCCGTGC ATAAGGCTGT GCTGNTCGAC GAGAAAGGGA CTGAAGCTGC TGGGGCCATG 60

## SEQ ID No 8b

SEQUENCE LENGTH: 54

SEQUENCE TYPE: Nucleotide

STRANDEDNESS: Single

TOPOLOGY: Linear

N: 3'-deoxyribofuranosyl naphthalene

GTGTCGTCCC GCAGTCAATG NNNNCCCACC TTCCCCTCTC TCCAGGCAAA TGGG

54

SEQ ID No 9

SEQUENCE LENGTH: 53

SEQUENCE TYPE: Nucleotide

5 STRANDEDNESS: Single

TOPOLOGY: Linear

N: 3'-deoxyribofuranosyl naphthalene

10 GTGTCGTCCC GCAGTCAATG NNNNGAGACT TGGTATTTTG TTCAATCATT AAG 53

15 SEQ ID No 10

SEQUENCE LENGTH: 54

SEQUENCE TYPE: Nucleotide

20 STRANDEDNESS: Single

TOPOLOGY: Linear

N: 3'-deoxyribofuranosyl naphthalene

25 AGTATGTATT ATGAGGACCG NNNNTGTCCA CGTGAGCCTT GCTCGAGGCC TGGG 54

30 SEQ ID No 11

SEQUENCE LENGTH: 54

SEQUENCE TYPE: Nucleotide

35 STRANDEDNESS: Single

TOPOLOGY: Linear

N: 3'-deoxyribofuranosyl naphthalene

40 TATAGGCAAG TAAGTTGATA NNNNTGGTGA TGATATCGTG GGTGAGTTCA TTTT 54

45 SEQ ID No 12

SEQUENCE LENGTH: 54

SEQUENCE TYPE: Nucleotide

50 STRANDEDNESS: Single

TOPOLOGY: Linear

N: 3'-deoxyribofuranosyl naphthalene

55 ACGATTGTTA ACCAAGATCT NNNNTGGTGA TGATATCGTG GGTGAGTTCA TTTA 54

SEQ ID No 13

SEQUENCE LENGTH: 30

SEQUENCE TYPE: Nucleotide

STRANDEDNESS: Single

TOPOLOGY: Linear

TTTTTTTTTT TATCAACTTA CTTGCCTATA

30

SEQ ID No 14

SEQUENCE LENGTH: 30

SEQUENCE TYPE: Nucleotide

STRANDEDNESS: Single

TOPOLOGY: Linear

TTTTTTTTTT AGATCTGGT TAACAATCGT

30

SEQ ID No 15

SEQUENCE LENGTH: 30

SEQUENCE TYPE: Nucleotide

STRANDEDNESS: Single

TOPOLOGY: Linear

TTTTTTTTTT CGGTCCTCAT AATACATACT

30

SEQ ID No 16

SEQUENCE LENGTH: 30

SEQUENCE TYPE: Nucleotide

STRANDEDNESS: Single

TOPOLOGY: Linear

TTTTTTTTTT AGTATGTATT ATGAGGACCG

30

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SEQ ID No 17  
 SEQUENCE LENGTH: 30  
 SEQUENCE TYPE: Nucleotide  
 5 STRANDEDNESS: Single  
 TOPOLOGY: Linear  
 TTTTTTTTTT TATAGGCAAG TAAGTTGATA 30  
 10

SEQ ID No 18  
 SEQUENCE LENGTH: 30  
 15 SEQUENCE TYPE: Nucleotide  
 STRANDEDNESS: Single  
 TOPOLOGY: Linear  
 20 TTTTTTTTTT ACGATTGTIA ACCAAGATCT 30

SEQ ID No 19  
 SEQUENCE LENGTH: 30  
 SEQUENCE TYPE: Nucleotide  
 25 STRANDEDNESS: Single  
 30 TOPOLOGY: Linear  
 TTTTTTTTTT CATTGACTGC GGGACGACAC 30

SEQ ID No 20  
 SEQUENCE LENGTH: 63  
 40 SEQUENCE TYPE: Nucleotide  
 STRANDEDNESS: Single  
 TOPOLOGY: Linear  
 N: thymidine phosphormorpholidate  
 45 AATTCCGTGC ATAAGGCTGT GCTGNNNNTC GACGAGAAAG GGACTGAAGC TGCTGGGGCC 60  
 ATG 63

50

55

SEQ ID No 21

SEQUENCE LENGTH: 60

SEQUENCE TYPE: Nucleotide

5 STRANDEDNESS: Single

TOPOLOGY: Linear

N: thymidine phosphormorpholidate

10 AATTCCGTGC ATAAGGCTGT GCTGNTCGAC GAGAAAGGGA CTGAAGCTGC TGGGGCCATG 60

SEQ ID No 22

15 SEQUENCE LENGTH: 60

SEQUENCE TYPE: Nucleotide

STRANDEDNESS: Single

20 TOPOLOGY: Linear

N: metaphosphate

AATTCCGTGC ATAAGGCTGT GCTGNTCGAC GAGAAAGGGA CTGAAGCTGC TGGGGCCATG 60

SEQ ID No 23

30 SEQUENCE LENGTH: 63

SEQUENCE TYPE: Nucleotide

STRANDEDNESS: Single

TOPOLOGY: Linear

35 N: metaphosphate

AATTCCGTGC ATAAGGCTGT GCTGNNNTCG ACGAGAAAGG GACTGAAGCT GCTGGGGCCA 60

TG 62

# Claims

- 45 1. A method for the amplification of a target nucleotide sequence, which method comprises contacting the target nucleotide sequence under hybridising conditions, together or sequentially, with a first primer for a desired portion thereof, a corresponding amplification primer, appropriate nucleotides and an agent for polymerisation of the nucleotides and the first primer comprising:-
- 50 1) a target binding nucleotide moiety which is substantially complementary to the desired portion of the target nucleotide sequence; and
- 2) a polynucleotide tail;
- such that the first primer may be subjected to primer extension whereby a first primer extension product is synthesised based on the target polynucleotide sequence as template, and after denaturation of the first
- 55 primer extension product from its template and hybridisation of the amplification primer to the desired portion of the first primer extension product, primer extension may be effected to form an amplification primer extension product, the presence of the first primer in the first primer extension product being effective to inhibit formation of a sequence complementary to the polynucleotide tail in the amplification

primer extension product.

2. A method as claimed in claim 1 wherein the amplification primer, in addition to the first primer, comprises:-

- 1) a target binding nucleotide moiety which is substantially complementary to the desired portion of the target nucleotide sequence; and
- 2) a polynucleotide tail;

such that the amplification primer may be subjected to primer extension whereby an amplification primer extension product is synthesised based on the first primer extension product as template, and after denaturation of the amplification primer extension product from its template and hybridisation of the first primer to the desired portion of the amplification primer extension product, primer extension may be effected to form a first primer extension product, the presence of the amplification primer in the amplification primer extension product being effective to inhibit formation of a sequence complementary to the polynucleotide tail in the first primer extension product.

3. A method as claimed in claim 1 or claim 2 wherein the first primer and/or the amplification primer comprise a target binding nucleotide moiety linked to a polynucleotide tail and wherein the target binding nucleotide moiety and the polynucleotide tail are in the opposite sense to one another.

4. A method as claimed in claim 1 or claim 2 wherein the first primer and/or the amplification primer comprise a target binding nucleotide moiety linked to more than one polynucleotide tail and wherein each polynucleotide tail is optionally linked to more than one target binding nucleotide moiety.

5. A method as claimed in claim 1 or claim 2 wherein the first primer and/or the amplification primer comprise a target binding nucleotide moiety and a polynucleotide tail with a nucleotide polymerisation blocking moiety therebetween.

6. A method as claimed in claim 5 wherein the nucleotide polymerisation blocking moiety comprises at least one ribofuranosyl naphthalene, deoxy ribofuranosyl naphthalene, metaphosphate or straight chain alkylene moiety or comprises at least two moieties independently selected from nucleotide methyl phosphonates and nucleotide phosphoramidates.

7. A method as claimed in claim 5 or claim 6 wherein the nucleotide polymerisation blocking moiety comprises at least two ribofuranosyl naphthalene, deoxy ribofuranosyl naphthalene or metaphosphate moieties, or comprises a C6-20 straight chain alkylene moiety.

8. A method as claimed in any one of claims 2-7 wherein the polynucleotide tail of a primer in respect of a first locus is distinguishable from the polynucleotide tail(s), in respect of a further locus or locus such that amplified product in respect of the first locus is captured onto a first and distinguishable solid phase whilst amplified product(s) in respect of a further locus or locus are captured on one or more further solid phase(s).

9. A method as claimed in any one of claims 2-7 wherein the amplified product in respect of a first locus is distinguishably labelled or marked via the primer's polynucleotide tail and the amplified product(s) in respect of a further locus or loci are also labelled or marked.

10. A method as claimed in claim 8 or claim 9 wherein the first and further locus correspond to alleles of a genetic locus.

11. A kit for the amplification of a target nucleotide sequence which comprises:-

a first primer and a corresponding amplification primer for each target nucleotide sequence to be amplified, each first primer comprising:-

- 1) a target binding nucleotide moiety which is substantially complementary to the desired portion of the target nucleotide sequence; and
- 2) a polynucleotide tail;

the presence of the first primer in the first primer extension product being effective to inhibit formation of a sequence complementary to the polynucleotide tail in the amplification primer extension product.

12. A kit as claimed in claim 11 wherein the amplification primer also comprises:-

- 1) a target binding nucleotide moiety which is substantially complementary to the desired portion of the target nucleotide sequence; and
- 2) a polynucleotide tail;

the presence of the amplification primer in the amplification primer extension product being effective to inhibit formation of a sequence complementary to the polynucleotide tail in a first primer extension product.

13. A kit as claimed in claim 11 or claim 12 which includes one or more of the following items:- optional internal control primers, appropriate different nucleotides and/or an agent for polymerisation of the nucleotides, and solid phase(s) for binding directly or indirectly to polynucleotide tails of the primers.

14. A kit as claimed in any one of claims 11-13 which includes solid phase in respect of each target nucleotide sequence to be amplified, each solid phase carrying a nucleotide sequence with at least a

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portion thereof substantially complementary to the polynucleotide tail of the relevant primer.

15. The use of a method or a kit as claimed in any one of the preceding claims for the detection of alleles indicative of an inherited disease or trait.

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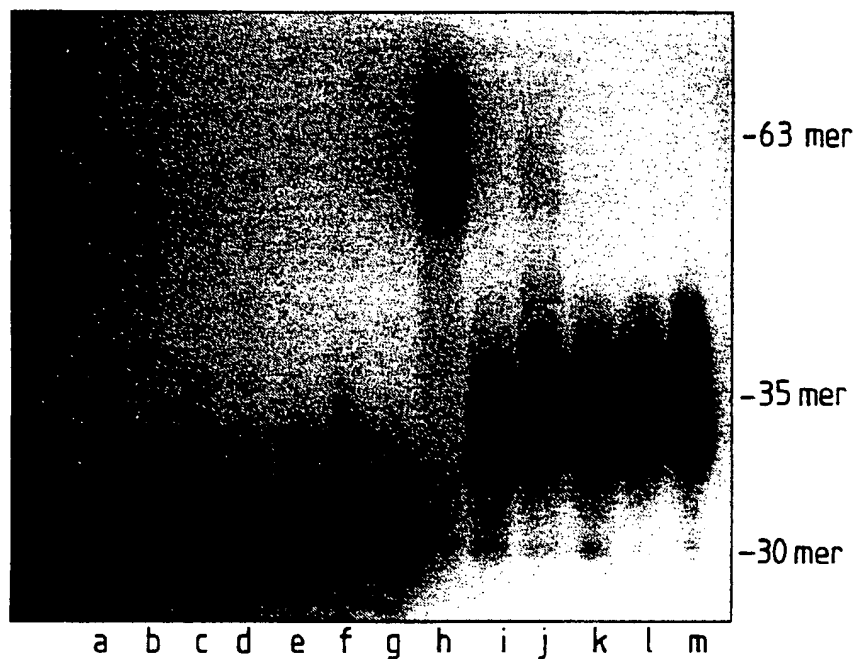
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55

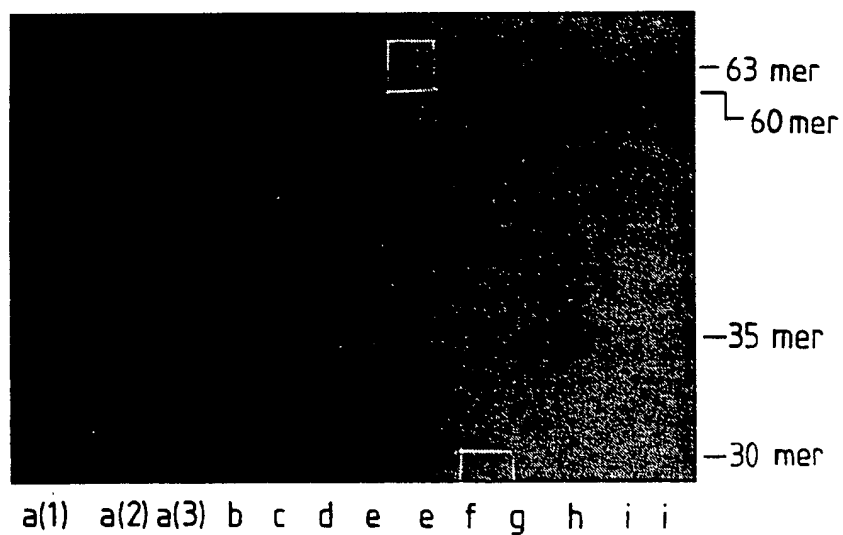
Nou eingereicht / Nov  
Nouvellement d'op

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*FIG. 1*

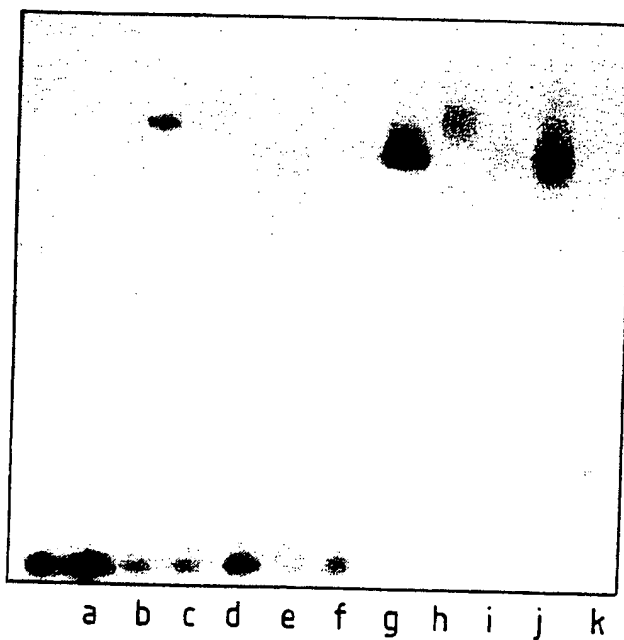


*FIG. 2*



Neu eingereicht / Ne  
Nouvellement déposé

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— 63 mer  
— 60 mer

*FIG. 3*

— 35 mer

— 30 mer

*FIG. 4*

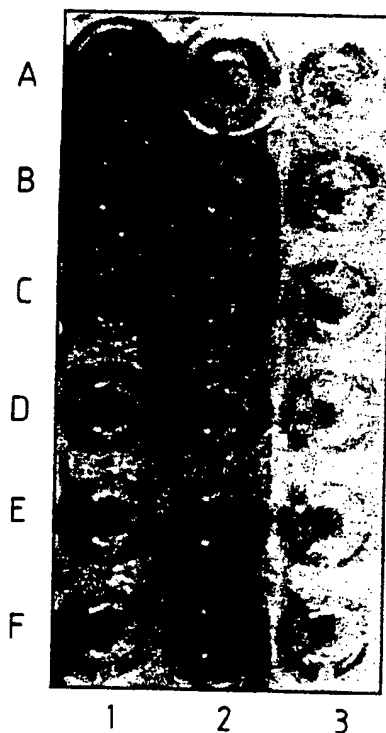
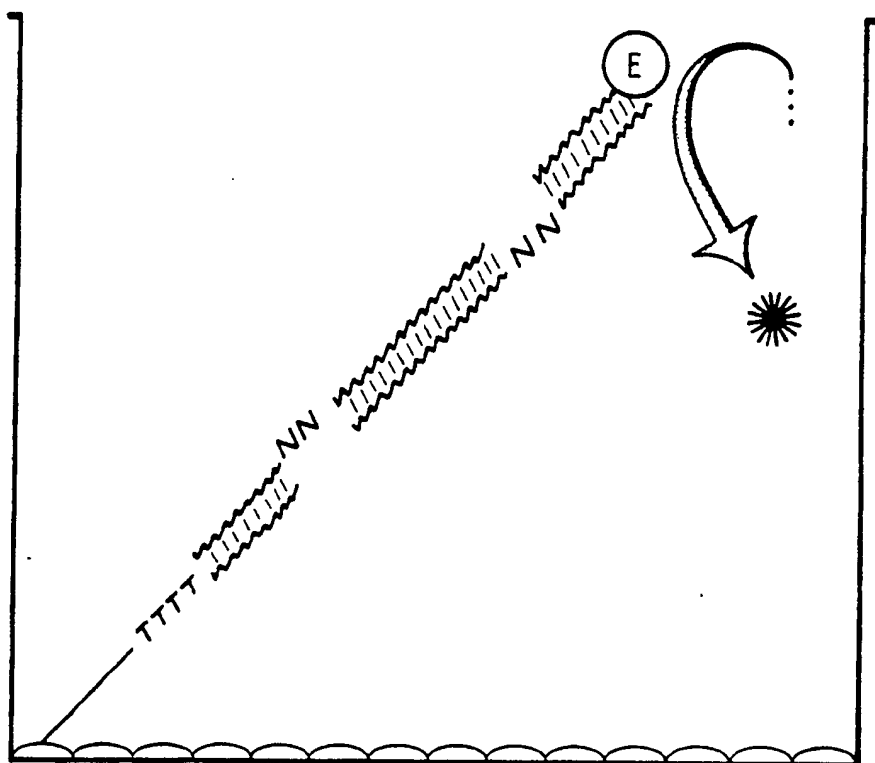
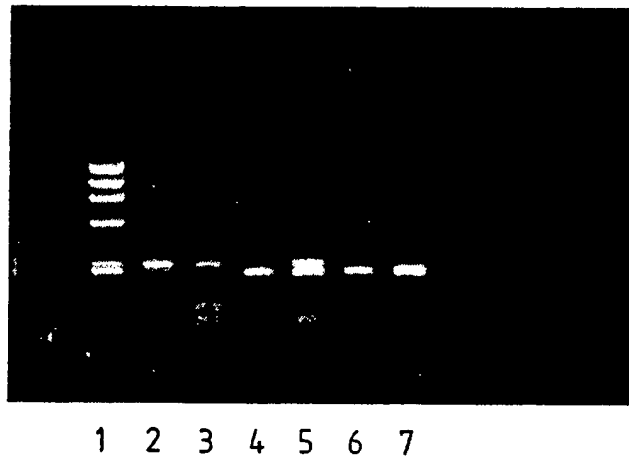


FIG. 5

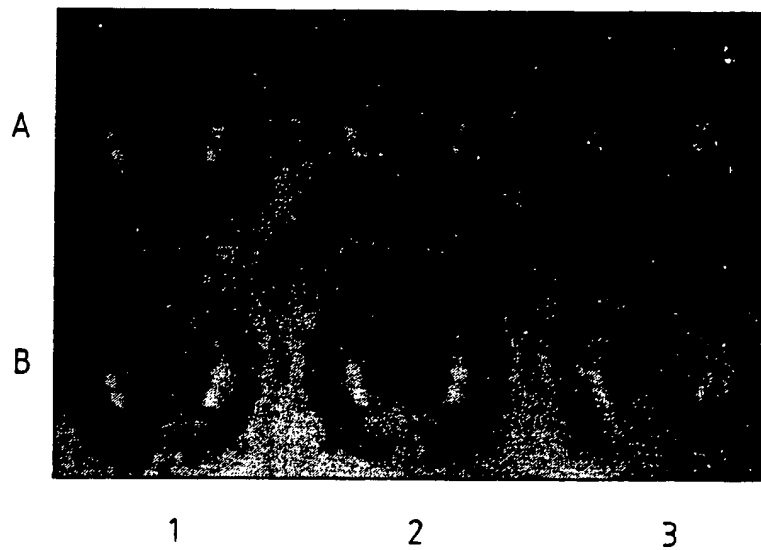


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Nouvellement déposé

*FIG. 6*



*FIG. 7*





Europäisches Patentamt  
European Patent Office  
Office européen des brevets



Publication number: **0 416 817 A3**

12

## EUROPEAN PATENT APPLICATION

21 Application number: **90309502.4**

51 Int. Cl.<sup>5</sup>: **C12Q 1/68, //C07H21/04**

22 Date of filing: **30.08.90**

30 Priority: **06.09.89 GB 8920097**

43 Date of publication of application:  
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84 Designated Contracting States:  
**AT BE CH DE DK ES FR GB GR IT LI LU NL SE**

88 Date of deferred publication of the search report:  
**04.09.91 Bulletin 91/36**

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**London SW1P 3JF(GB)**

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**Welwyn Garden City Hertfordshire, AL7**  
**1HD(GB)**

54 **Amplification processes.**

57 A method for the amplification of a target nucleotide sequence using a first primer comprising 1) a target binding nucleotide moiety which is substantially complementary to the desired portion of the target nucleotide sequence; and 2) a polynucleotide tail; the first primer being such that whilst an extension product thereof may itself serve as a template for primer extension to form an amplification primer extension product, formation of an amplification primer extension product comprising a sequence complementary to the polynucleotide tail is inhibited.

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European  
Patent Office

## EUROPEAN SEARCH REPORT

Application Number

EP 90 30 9502

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	GB-A-2 202 328 (ORION-YHTYMA OY) * Page 6, paragraphs 1,2 * - - -	1,2,11,12	C 12 Q 1/68 // C 07 H 21/04
D,A	AGR. BIOL. CHEM., vol. 36, no. 9, 1972, pages 1651-1653; H. OHRUI et al: "Facile synthesis of C-glycosides of aromatic compounds" * Whole document * - - -	5-7	
D,A	NUCLEIC ACIDS RESEARCH, vol. 12, no. 11, 1984, pages 4539-4557, IRL Press Ltd, Oxford, GB; N.D. SHINHA et al.: "Polymer support oligonucleotide synthesis XVIII 1,2): use of beta-cyanoethyl-N,N-dialkylamino-/N-morpholino phosphoramidite of deoxynucleosides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product" * Whole document * - - -	5-7	
D,A	NUCLEIC ACIDS RESEARCH, vol. 17, no. 7, 11th April 1989, pages 2503-2516, Eynsham, Oxford, GB; C.R. NEWTON et al.: "Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS)" * Whole document * - - -	10,15	
D,A	EP-A-0 237 362 (CETUS CORP.) - - -		TECHNICAL FIELDS SEARCHED (Int. Cl.5)  C 12 Q
P,A	GB-A-2 221 909 (IMPERICAL CHEMICAL IND.) * Pages 22,49,50; claim 5 * - - -	1,2,5,11, 12	
T	WO-A-9 000 372 (COLLABORATIVE RESEARCH) * Whole document * - - - - -	1,8,10,11, 15	
The present search report has been drawn up for all claims			
Place of search  The Hague		Date of completion of search  06 June 91	Examiner  MOLINA GALAN E.
<div>CATEGORY OF CITED DOCUMENTS</div> <div>X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document T: theory or principle underlying the invention</div> <div>E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons - - - - - &amp;: member of the same patent family, corresponding document</div>			